# Effects of metal pollution across different levels of biological organisation in the River Rhine system with a special focus on *Theodoxus fluviatilis* as a potential indicator organism

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Louisa Marie Rothmeier

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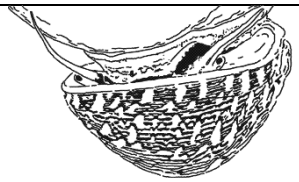

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The crucial first step to survival
in all organisms is habitat selection.
If you get to the right place,
everything else is likely to be easier.

Edward O. Wilson

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## Abstract

Pollution through anthropogenic activities is a major threat for freshwater ecosystems. Metals are a relevant group of contaminants that affect aquatic organisms, communities, and ecosystems. This thesis aims at examining the effects of metal pollution across different levels of biological organisation in the anthropogenically degraded environment of the German River Rhine system and at discussing the role of the freshwater snail *Theodoxus fluviatilis* as an indicator organism. A field investigation to detect the effects of metal pollution at the macroinvertebrate community level in the River Rhine system shows that the measured metals are less relevant than geogenic originated factors at the sampling sites (paper 1). Additionally, the community structure is strongly characterised by a separation of non-native taxa in the River Rhine and native taxa in its tributaries. This indicates that the occurrence of invasive species could possibly be a factor structuring the macroinvertebrate community to such a high extent that the effects of other stressors are masked. Regarding the effects of metal pollution on the population, organism, and physiological level of the Danubian form of *T. fluviatilis* in the River Rhine, only the metal chromium affects the snail specimens negatively (paper 2). As the Danubian *T. fluviatilis* seems to be able to cope with the partly high concentrations of copper and zinc at the sampling sites, its suitability as an indicator for metal pollution is questionable. However, in the practice of Rhine monitoring, a distinction between the two occurring, phylogenetically different forms of *T. fluviatilis* is not made, though the original, native indicator has been replaced by the presumably more tolerant non-native Danubian form. An intraspecific variability in *T. fluviatilis* is assumed, for what first indications are found regarding differences in the infection of the two forms with parasitic trematodes (paper 3). The investigations about a possible intraspecific variability have to be complemented by comparative laboratory analyses, for which purpose an experiment with the two forms of *T. fluviatilis* is developed (paper 4). The results of this thesis show that investigations about the effects of pollutants in ecosystems should encompass a variety of responses across different levels of biological organisation, as well as the co-occurrence and interaction of various abiotic and biological stressors. Further, a possible intraspecific variability in the indicator organism *T. fluviatilis* has to be included in the monitoring strategies and ecological assessment of the anthropogenically degraded River Rhine system.

## Zusammenfassung

Verschmutzung durch anthropogene Aktivitäten stellt eine große Bedrohung für Süßwasserökosysteme dar. Metalle sind eine relevante Gruppe von Schadstoffen, die aquatische Organismen, Gemeinschaften und Ökosysteme beeinträchtigen. Ziel dieser Arbeit ist es, die Auswirkungen von Metallverschmutzung auf verschiedenen Ebenen der biologischen Organisation in der anthropogen degradierten Umwelt des deutschen Rheinsystems zu untersuchen und die Rolle der Süßwasserschnecke *Theodoxus fluviatilis* als Indikatororganismus zu diskutieren. Eine Felduntersuchung zu den Auswirkungen von Metallverschmutzung auf der Ebene der Makrozoobenthosgemeinschaft im Rheinsystem zeigt, dass die gemessenen Metalle an den untersuchten Standorten von geringerer Relevanz als geogene Faktoren sind (Paper 1). Zusätzlich ist die Gemeinschaftsstruktur stark durch eine Trennung von gebietsfremden Taxa im Rhein und heimischen Taxa in den Nebenflüssen gekennzeichnet. Insofern könnte das Vorkommen invasiver Arten im Rhein ein Faktor sein, der die Makrozoobenthosgemeinschaft so stark strukturiert, dass die Effekte anderer Stressoren überlagert werden. Hinsichtlich der Auswirkungen von Metallen auf Populations-, organismischer und physiologischer Ebene der Donauform von *T. fluviatilis* im Rhein werden lediglich negative Auswirkungen von Chrom gefunden (Paper 2). Da diese Form der Art die teilweise hohen Konzentrationen von Kupfer und Zink an den Probenahmestellen zu tolerieren scheint, ist ihre Eignung als Indikator für Metallbelastung fraglich. Jedoch wird in der Rheinmonitoringpraxis nicht zwischen den beiden vorkommenden, phylogenetisch unterschiedlichen Formen von *T. fluviatilis* unterschieden, obwohl der ursprüngliche, einheimische Indikator durch die vermutlich tolerantere, gebietsfremde Donauform ersetzt wurde. Eine intraspezifische Variabilität bei *T. fluviatilis* wird vermutet, wofür erste Hinweise in der unterschiedlichen Infektion der beiden Formen mit parasitären Trematoden gefunden werden (Paper 3). Die Untersuchungen zur möglichen intraspezifischen Variabilität müssen durch vergleichende Laboranalysen ergänzt werden, wozu ein Experiment mit den beiden Formen von *T. fluviatilis* entwickelt wird (Paper 4). Die Ergebnisse dieser Arbeit zeigen, dass Untersuchungen zu den Auswirkungen von Schadstoffen in Ökosystemen eine Vielzahl von Reaktionen auf verschiedenen Eben der biologischen Organisation sowie das gleichzeitige Auftreten und die Wechselwirkungen verschiedener abiotischer und biologischer Stressoren umfassen sollten. Ferner muss eine mögliche intraspezifische Variabilität beim Indikatororganismus *T. fluviatilis* in die Monitoringstrategien und ökologische Bewertung des anthropogen degradierten Rheinsystems einbezogen werden.

## List of papers

**Paper 1**

Is metal pollution a relevant environmental factor affecting the macroinvertebrate community in the River Rhine system? Louisa Marie Rothmeier, Andreas Martens, Karsten Grabow, Jennifer Bartz, and René Sahm. Manuscript.

**Paper 2**

The Danubian cryptic invader *Theodoxus fluviatilis* (Gastropoda: Neritidae) in the River Rhine: a potential indicator for metal pollution? Louisa Marie Rothmeier, Andreas Martens, Burkard Watermann, Karsten Grabow, Jennifer Bartz, and René Sahm. Ecotoxicology (2022) 31:24-32. https://doi.org/10.1007/s10646-021-02485-4.

**Paper 3**

The Ponto-Caspian parasite *Plagioporus* cf. *skrjabini* reaches the River Rhine system in Central Europe: higher infestation in the native than in the introduced Danubian form of the gastropod *Theodoxus fluviatilis*. Louisa Marie Rothmeier, René Sahm, Burkard Watermann, Karsten Grabow, Meike Koester, Anna Cichy, and Andreas Martens. Hydrobiologia (2021) 848:2569-2578. https://doi.org/10.1007/s10750-021-04578-x.

**Paper 4**

Evaluation of a preliminary test design for a growth experiment with juveniles of two phylogenetic different forms of the freshwater snail *Theodoxus fluviatilis*. Louisa Marie Rothmeier, Andreas Martens, Burkard Watermann, and René Sahm. Manuscript.

**Author contributions**

In writing the four papers, I took the leading role. The general creation of concepts and aims for the investigations and studies was largely carried out by myself together with René Sahm and Andreas Martens. All four manuscripts were carefully read by all co-authors and changes and revisions were made based on detailed discussions among the authors. The field work of sample collection for **papers 1**, **2**, and **3** was conducted by myself. I also carried out the species determination of the sampled macroinvertebrates for **paper 1** and the preparation of parasites from *T. fluviatilis* for **paper 3**, with the support of Karsten Grabow's expert

knowledge. The sampling of snail specimens of the Northern-European form of *T. fluviatilis* for **paper 4** as well as the laboratory work of determining the periphyton biomass for **papers 1** and **2** was conducted by René Sahm at the German Environment Agency in Berlin. The measurements of metal concentrations in water and biofilm samples for **papers 1** and **2** were carried out by Jennifer Bartz, also at the German Environment Agency. Burkard Watermann at LimnoMar in Hamburg, Germany, conducted the histopathological examination of snail specimens for **papers 2**, **3**, and **4** and supported my work on all manuscripts with his huge expertise on the histopathology of invertebrates and on the influence of metal pollution in aquatic environments. The genetic analyses and examination of *T. fluviatilis* specimens for **paper 3** was conducted by Meike Koester and myself at the Koblenz Campus of the University of Koblenz-Landau. Meike Koester also strongly supported the writing of **paper 3** with her expertise on genetic analyses and the conduction of ecological studies. The laboratory and genetic examination of parasites derived from *T. fluviatilis* was carried out by Anna Cichy at the Nicolaus Copernicus University in Torun, Poland. Anna Cichy also strongly supported the work on **paper 3** with her expertise in the field of mollusc parasitology and the connections to environmental and ecological issues. The laboratory growth experiment for **paper 4** was conducted by myself, while René Sahm supported me in the creation of the experimental design, the implementation of the laboratory methods, and the procurement of the materials. The data evaluations and statistical analyses of all four papers were conducted by myself and carefully discussed in consultation with René Sahm.

## Paper aims

Among the multitude of stressors affecting aquatic ecosystems worldwide, metals are a highly relevant group of contaminants affecting aquatic biota and impairing freshwater habitats. The general aim of this thesis is to examine the effects of metal pollution on different levels of biological organisation in the anthropogenic degraded environment of the German River Rhine system. Further, the role of the often-used indicator organism *T. fluviatilis* is discussed in light of the actual situation of the native form of the species having been replaced by the Danubian form in the River Rhine. The specific paper aims (Table 1) contribute to these general objectives.

**Table 1**: Specific aims of the four papers included in this thesis.

| Paper | Aims |
|---|---|
| 1 | Examine if metal pollution is a relevant stressor affecting the macroinvertebrate community in the River Rhine system. |
| 2 | 1. Determine the effects of metal pollution on the Danubian form of *T. fluviatilis* in the River Rhine.<br>2. Discuss the potential of the Danubian form of *T. fluviatilis* as an indicator organism for metal pollution. |
| 3 | Compare specimens of the Danubian form of *T. fluviatilis* from the River Rhine and of the Northern-European form from three tributaries histopathologically and examine differences regarding parasitation and pathologic organic alterations. |
| 4 | Conduct and evaluate a test design for a laboratory 40-days growth experiment with juveniles of the Danubian and Northern-European form of *T. fluviatilis*:<br>1. Examine if the snails significantly grow over experimental time.<br>2. Analyse differences between the two snail forms regarding survival, growth, activity, and histopathology. |

## Methods

The four papers included in this thesis are based on three field studies (**papers 1 - 3**) and one experimental study (**paper 4**) in which different field, analytical, laboratory, experimental, and statistical methods are applied (Table 2). All statistical analyses were conducted with the program R (R Core Team 2021). For details, full descriptions, and references of the used methods, see the respective paper.

**Table 2**: Field, analytical, experimental, and statistical methods that are used in the papers included in this thesis.

**A. Field methods**

| *Paper(s) no.* | *Objective* | *Description* |
|---|---|---|
| 1, 2 | Water sample collection | Samples were transported in polypropylene bottles, filtrated on 0.45 µm, and for metal analyses acidified with ultrapure nitric acid to a concentration of 0.5%. |
| 1, 2 | Biofilm sample collection | Periphyton was scraped from stones with a spatula, stored in river water, and acidified with ultrapure nitric acid to a concentration of 0.5%. |
| 1, 2, 3, 4 | Sampling of *T. fluviatilis* | Snails were carefully removed from stones, eggs on shells were counted, shell length was measured with a digital calliper, then snails were transferred to polypropylene bottles with river water (for dissection, histopathology, or experiment), or pure 96% ethanol (for genetic analysis). |
| 1 | Sampling of macroinvertebrates | Macroinvertebrates were carefully brushed from stones, collected with a sieve (200 µm mesh size), and transferred to polypropylene bottles with pure 96% ethanol. |
| 1, 2 | Determination of stone surface | Stones were wrapped in aluminium foil and the surface was determined by weighing reference foil pieces and calculating the function of foil weight to surface. |

**B. Analytical and laboratory methods**

| *Paper(s) no.* | *Analyses* | *Technique / Instruments* |
|---|---|---|
| 1, 2 | Water temperature, dissolved oxygen, pH, electrical conductivity | DO-100, PH-100 ATC, LWT-01 |
| 1, 2 | Nutrients in water: $Cl^-$, $NO_2^-$-N, $NO_3^-$-N, $PO_4^{3-}$-P, $SO_4^{2-}$, $NH_4^+$-N, $Ca^{2+}$, $Mg^{2+}$, $Na^+$, Si | Continuous-flow-analysis, ion chromatography |

| | | |
|---|---|---|
| 1, 2 | Metals in water: Cu, Cr, Sr, Zn, Fe | Inductively coupled plasma optical emission spectrometry (ICP-OES) |
| 1, 2 | Metals in biofilm: Cu, Cr, Sr, Zn, Fe | ICP-OES |
| 1, 2 | Biomass of biofilm | Determination of ash-free dry mass (AFDM): sample filtration on precombusted glass-fibre filters, drying at 105 °C for 24 h, weighing, combusting at 550 °C for 8 h, weighing, AFDM-calculation by subtraction |
| 1 | Identification of macroinvertebrates | Microscopic identification using an EZ4 microscope (Leica) with 8x magnification and standard macroinvertebrate identification literature |
| 2, 3, 4 | Histopathology of *T. fluviatilis* | Routine histological preparation, counter-staining with haematoxylin and eosin, microscopic observations (determination of sex, pathological alterations in organs) |
| 3 | Sampling of parasites from *T. fluviatilis* | Microscopic dissection of snails, parasite sampling from the snail's visceral bags, storing in pure 96% ethanol |
| 3 | Genetics of *T. fluviatilis* and parasites: mitochondrial COI | DNA extraction, Polymerase chain reaction (PCR), sequencing, edition and alignment of sequences with Geneious 11.1.2 (*T. fluviatilis*) and CAP3 (parasites) |

**C. Experimental methods**

| *Paper no.* | *Objective* | *Description* |
|---|---|---|
| 4 | Growth experiment | Juveniles of the Danubian and Northern-European form of *T. fluviatilis* were held in the laboratory under natural feeding and constant conditions for 40 days. The parameters growth, survival, and activity were analysed. Post-experimental histopathological analysis was conducted. |

**D. Statistical methods**

| *Paper no.* | *Analyses* |
|---|---|
| 1 | Distance-based redundancy analysis (db-RDA), *ordiR2step()* variable selection, *anova()* permutation tests, Generalised linear model (GLM), Permutational multivariate analysis of variance (PERMANOVA), *simper()* (similarity percentages) analysis |
| 2 | Db-RDA, *bioenv()* variable selection, *anova()* permutation tests, GLM |
| 3 | Maximum likelihood analysis, PERMANOVA, *simper()* analysis, Mann-Whitney-U-test |
| 4 | Cox proportional hazards model, GLM, Mann-Whitney-U-test |

## The freshwater snail *Theodoxus fluviatilis*

The species *Theodoxus fluviatilis* (Linné, 1758) of the gastropod family Neritidae is widely distributed and the most abundant species of the genus *Theodoxus* in Central Europe (Bunje 2005, Glöer 2015, Zettler 2008). It is rather variable in shell pattern and coloration, with a maximum shell length of 13 mm and a relatively short body protruding barely from the shell while crawling (Glöer 2015, Zettler et al. 2004, Figure 1). *T. fluviatilis* is dioecious and sexually reproducing. It lays yellowish egg capsules of about 1 mm in diameter containing 30-70 eggs and attaches them on hard substrate or shells of other snails (Glöer 2015, Orton and Sibly 1990). From the capsules, one embryo develops and the juvenile snail hatches as a miniature adult without larval stage whilst the other eggs serve as food (Glöer 2015, Orton and Sibly 1990).

*T. fluviatilis* is a nearly exclusive grazer and feeds on biofilm by scraping algae with its radula from hard substrate (Glöer 2015, Neumann 1959, Skoog 1978), which is necessary to crush diatoms mechanically to make them available for digestion (Neumann 1961). Hence, the preferred habitat and abundance of *T. fluviatilis* in the littoral, stony zone of streams and lakes is presumed to be a consequence of the nutritional physiology of the species and its demands on hard substrate as a counterpart for the radula for grazing on diatoms (Neumann 1961, Zettler et al. 2004). If its substrate demands are met, *T. fluviatilis* is able to colonise different habitats like springs, streams, rivers, lakes, and even brackish water (Bunje 2005, Zettler 2008).

Phylogeographically, the ancestral range of *T. fluviatilis* is hypothesised to be in the Ponto-Pannonian region in the area of southern Ukraine, Romania, and Hungary, from where the species colonised Europe (Bunje 2005). By analysing the mitochondrial cytochrome c oxidase subunit I (COI) gene of populations all over Europe, Bunje (2005) found clear geographical subdivisions of the species, particularly between Eastern and Western Europe and separating Northern-European populations. In Germany, populations of *T. fluviatilis* which are native to all bigger rivers, e. g. Rhine, Main, Moselle, Neckar, and Elbe, can be phylogenetically allocated to the Northern-European group of the species (Bunje 2005). In the last few decades, these populations showed a decline in abundance, starting from industrial used rivers (Westermann et al. 2007, Zettler 2008). The decreasing trend was especially recognised in the River Rhine, where *T. fluviatilis* has always been a typical macroinvertebrate species and plays an

important role as an indicator organism for river monitoring (Gergs et al. 2015, Westermann et al. 2007, Zettler 2008). As *T. fluviatilis* was assumed to be extinct in the Upper River Rhine by the end of the 20th century, a lot of attention was paid to the rediscovery of the snail near the mouth of the river Main in the year 2006 (IKSR/CIPR/ICBR 2015, Westermann et al. 2007). Though, the newly discovered specimens in the River Rhine do not only differ morphologically from the native Northern-European form of *T. fluviatilis* (Figure 2), but also genetically, as analyses showed that they carry the Danubian haplotype known from populations in the river Danube and the Ukraine Black Sea basin (Gergs et al. 2015). Therefore, it is assumed that recent River Rhine populations originate from specimens of the Danubian form of *T. fluviatilis* that have been transported, probably by ships as vectors, from the Black Sea region through the Main-Danube canal into the River Rhine (Gergs et al. 2015). The Danubian form colonises the River Rhine along several hundred kilometres showing partly high densities (Gergs et al. 2015, Rothmeier and Martens 2019, Westermann et al. 2007) and has recently been found for the first time in the High Rhine (Baur et al. 2022). The native Northern-European form of *T. fluviatilis* can still be found in smaller tributaries of the River Rhine system, e. g. the rivers Kocher, Jagst, and Tauber.

The establishment of the Danubian form of *T. fluviatilis* in the River Rhine can be noted as an example for intraspecific cryptic invasion, which is defined as the invasion of another lineage of a species into an area where a different local lineage of the same species already existed (Gergs et al. 2015, Morais and Reichard 2018). Intraspecific cryptic invasion is often triggered by anthropogenic effects, e. g. the elimination of natural barriers between originally separate river basins by navigation canals (Morais and Reichard 2018). The opening of the Main-Danube canal in 1992 is an example for such an event, connecting the Black Sea region to the River Rhine, which resulted in the occurrence of the Danubian cryptic invader *T. fluviatilis* in the River Rhine.

**Figure 1:** Adult specimen of the freshwater snail *Theodoxus fluviatilis* (Danubian form) in the River Rhine. Photograph by K. Grabow.


**Figure 2:** Shells of adult individuals from two phylogenetically different forms of *Theodoxus fluviatilis*: the Northern-European form (left) and the Danubian form (right). Photograph by L. Rothmeier.

# 1 General background and aims

The growing concerns about the effects of environmental toxicants on species other than humans resulted in the introduction of the term "ecotoxicology" together with the development of the corresponding discipline (Walker et al. 2012). The field of ecotoxicology represents a molecules-to-ecosystems approach, as it focuses on harmful effects of pollutants on all levels of biological organisation (Fent 2013, Walker et al. 2012, Figure 3). First, it deals with the molecular structure of pollutants, their concentrations and movements in air, water, soils, sediments, and through food chains, with chemical transformation and biotransformation (Walker et al. 2012). Second, the effects of pollutants on individual organisms are analysed regarding molecular, cellular, and whole animal responses (Fent 2013, Walker et al. 2012). Finally, ecotoxicology aims at answering the question about the effects of pollutants at the levels of populations, communities, and whole ecosystems (Walker et al. 2012).

The main objectives of this thesis are to examine the effects of metal pollution in the River Rhine system and to discuss the role of the freshwater snail *T. fluviatilis* as an indicator organism in light of the special situation of the native Northern-European form having been replaced by the Danubian form in the River Rhine. The subtopics and papers that contribute to the overall objectives can be allocated to the respective levels of biological organisation considered in ecotoxicology (Figure 3):

- At the **pollutant** level, the focus lies on investigating the effects of metal pollution in the River Rhine system (**papers 1** and **2**) and on examining its interactions with other abiotic and biological stressors (**papers 1**, **2**, and **3**).
- **Physiological changes**, **organism responses**, and **population changes** are analysed using *T. fluviatilis* as a model organism (**paper 2**). Intraspecific differences between the Northern-European and the Danubian form of *T. fluviatilis* regarding infestations of parasites and physiological organic alterations are examined on the basis of histopathological investigations of specimens from the field (**paper 3**). In light of possible intraspecific differences in *T. fluviatilis*, which imply the question if they could also exist in the sensitivity towards toxicants, the role and suitability of this species as an indicator organism for environmental monitoring is evaluated. A first step towards

a comparison of the two snail forms under laboratory conditions is done by conducting and evaluating a test design for a growth experiment with juveniles of *T. fluviatilis* (**paper 4**).

- At the **community** level of biological organisation, the effects of metal pollution on the macroinvertebrate community in the River Rhine and three tributaries are analysed (**paper 1**).
- On top of the stair chart, the focus lies on **ecosystems**. The observations in this thesis move in the field of freshwater ecology and all field investigations are conducted in the environment of the River Rhine system in Germany (**papers 1**, **2**, and **3**).

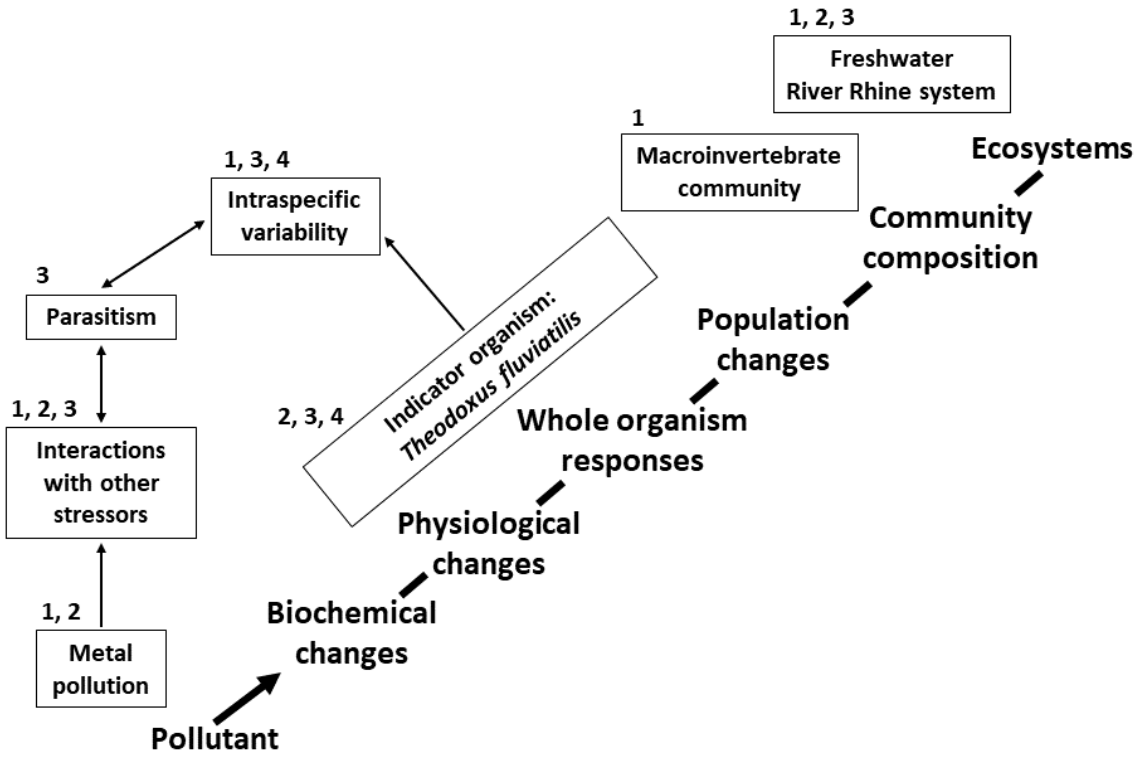


**Figure 3:** Fields of interest in ecotoxicology as a molecules-to-ecosystems approach across different levels of biological organisation (stair chart, modified after Walker et al. 2012). The boxes show the allocation of the central themes of this thesis to the respective levels and the numbers show the papers in which the themes are addressed.

# 2 Effects of metal pollution in the River Rhine

Freshwater ecosystems contribute to a large extent to the global biodiversity and are simultaneously highly threatened by deterioration through a multitude of stressors associated with human activities including habitat degradation, pollution by a wide range of chemicals, and biological invasions (Carpenter et al. 2011, Ormerod et al. 2010, Schäfer et al. 2016, Vörösmarty et al. 2010). Regarding the contamination of freshwater ecosystems by chemical substances, many researchers highlight the importance of metal pollution (Carpenter et al. 2011, Daehne et al. 2017, Mussali-Galante et al. 2013, Rainbow 2002). Though metals are natural substances and have been present on earth since its formation, they are usually considered as pollutants (Fent 2013). In most cases, they become harmful through human activities like mining, smelting, or the use in biocidal products, which relocate them to the environment where they can have lethal or sublethal effects on living organisms (Fent 2013, Mussali-Galante et al. 2013, Sures 2008, Walker et al. 2012). In an exposed organism, the toxicity of a metal depends on threshold concentrations in relation to its particular physiology, as to whether the respective metal is used for metabolic purpose, excreted, stored in the body, or exerting toxic effects (Rainbow 2002). Some organisms are able to detoxify metal ions by binding them to proteins like metallothionein, or by depositing them in insoluble forms in intracellular granules for long-term storage or excretion in the feces (Walker et al. 2012). Of especially great relevance are metals that are non-essential for the metabolic activity of organisms. These are toxic at quite low concentrations and have to be excreted, e. g. mercury, chromium, and cadmium. Further, there are metals that are essential to living organisms but become toxic at high concentrations, e. g. iron, copper, and zinc (Mussali-Galante et al. 2013, Rainbow 2002).

Metals are not biodegradable and persist in the environment. Chronic environmental exposure leads to various responses across all levels of biological organisation, from alterations in molecules to individual diseases and threats to ecosystem integrity (Fent 2013, Mussali-Galante et al. 2013). This thesis aims at examining the effects of metal pollution on different levels of biological organisation (Figure 3), regarding effects on the macroinvertebrate community level in **paper 1** and on the population, physiological, and organism level of the freshwater snail *T. fluviatilis* in **paper 2** (aim 1).

All field examinations in this thesis focus on the heavily anthropogenically influenced environment of the River Rhine system. Linking the countries Switzerland, Liechtenstein, Austria, Germany, France, and the Netherlands, the River Rhine is the main inland waterway of one of the most important economic regions of Europe (Leuven et al. 2009). The dense population and heavy industrialisation lead to an enormous influence of human activities on its catchment area and to a relevant environmental deterioration (Leuven et al. 2009, Malle 1990). Although pollution in the River Rhine and its tributaries is decreasing, contaminants which are considered to have negative effects on the drinking water quality and the ecological status are still found (IKSR/CIPR/ICBR 2021b). Of these contaminants, metals play an important role as priority substances in the context of the water quality assessment based on the Water Framework Directive (WFD) (European Community 2000, IKSR/CIPR/ICBR 2021b). The WFD was adopted in 2000 to require the member states of the European Union to achieve or preserve a "good status" of their surface waters by 2015 or by 2027 at the latest (European Community 2000, Hering et al. 2010, Lagerström et al. 2020, Schäfer et al. 2016). It aims at assessing improvements of ecological quality by the response of organisms (such as fish, invertebrates, or macrophytes) or biotic communities to stressors and considers the presence and intensity of contaminants as supporting monitoring elements (Hering et al. 2010, Schäfer et al. 2016). The "good status" is reached when both good ecological and good chemical status are achieved (Lagerström et al. 2020). This is not yet the case for the River Rhine, as e. g. target values for metals are still exceeded at single locations (IKSR/CIPR/ICBR 2021b, IKSR/CIPR/ICBR 2018, Sjerps et al. 2017). On the spatial scale, there are differences between more and less polluted sites (IKSR/CIPR/ICBR 2021b), whereas one can suppose that there are certain hotspots of metal contamination, like close to industrial plants, in harbours, and in marinas (Daehne et al. 2017, Lagerström et al. 2020, Rautengarten 1993).

Although bordering countries conduct a permanent biological and chemical monitoring of i. a. metal concentrations in the River Rhine, the respective results are presented in separate reports (IKSR/CIPR/ICBR 2021a, IKSR/CIPR/ICBR 2021b). The assessment of the chemical status results from punctual measurements of a selection of priority pollutants, which does neither reflect the dynamics of pollutants in the river and their potential ecological effects, nor the effects of contaminants in realistic exposure scenarios (Tlili et al. 2016). But, investigating the effects of priority pollutants like metals at different sites on biological variables like species composition, population changes, or individual organism responses is

essential to assess the importance of metal pollution. For this purpose, multivariate analyses based on field measurements at less polluted sites as well as hotspots, linking the responses of the river biota to metal pollution, are necessary. **Paper 1** within the framework of this thesis is a multivariate field study examining the effects of metal pollution on the macroinvertebrate community level in the River Rhine system. **Paper 2** aims at investigating the effects of metal pollution on the population, physiological, and organism level of a particular species, the freshwater snail *T. fluviatilis* (aim 1).

In the practice of water quality assessment, concentrations of metals are usually measured in water samples, or in sediments (Fent 2013). Metal concentrations in periphyton are often neglected, though biofilms are recognised by the WFD as a necessary target to be considered in river assessment (Sabater et al. 2007). The biofilm as a community of (micro)organisms on surfaces is one of the first biological compartments which absorb and accumulate dissolved toxicants, and integrates them in the food chain by being eaten by consumers (Bhaskar and Bhosle 2006, Morin et al. 2008, Sabater et al. 2007). Hence, periphyton metal contamination is of a great importance for aquatic organisms in rivers and streams, especially for grazers which feed on biofilms (Sabater et al. 2007). Both **paper 1** and **2** of this thesis do not only consider metal concentrations in water samples, but also in biofilm samples from the respective study sites to be included in the analyses.

There are a multitude of studies about the different responses of aquatic organisms to metal exposure, e. g. for daphnia (Bellavere and Gorbi 1981, Meyer et al. 2015), gastropods (Brix et al. 2011, Cain et al. 2016, Gao et al. 2017, Khangarot and Das 2010), and fish (Bellavere and Gorbi 1981, Hansen et al. 2007). Though, an approach which encompasses a range of different freshwater taxa forming the macroinvertebrate community is essential to analyse the effects of metal pollution on this level of biological organisation in the River Rhine. In **paper 1**, relationships between aqueous and biofilm concentrations of metals together with various other anthropogenic and geogenic originated stressors and the densities of 25 macroinvertebrate taxa are analysed at 30 sites in the German River Rhine and three of its tributaries, the rivers Jagst, Kocher, and Tauber. It is hypothesised that metal pollution is a relevant factor structuring the macroinvertebrate community in the River Rhine system. However, the results of **paper 1** show that among the set of the analysed environmental factors, the measured metals are of less importance for the macroinvertebrate community than the geogenic originated factors at the sampling sites, which are the concentrations of

calcium, sulphate, and strontium. Furthermore, there is a significant separation of the communities between the River Rhine, which is characterised by non-native taxa, and the tributaries, where mainly native taxa occur. It is suggested that the occurrence of non-native species as a biological stressor masks a possible effect of metal pollution, at least for the macroinvertebrate taxa at the analysed sampling sites. These findings reflect the situation present in the anthropogenic influenced environment of the River Rhine system, i. e. a co-occurrence and interaction of a variety of abiotic and biological stressors, which has to be considered in river monitoring.

**Paper 2** is the first field study which examines the effects of metal pollution in the River Rhine on a freshwater invertebrate grazer regarding the physiological, organism and population level (aim 1). The Danubian form of *T. fluviatilis* is used for the analyses, as the freshwater snail is exposed to contaminants through two pathways: the respiratory route, due to its uptake of pollutants dissolved or suspended in ambient water, and the dietary route, due to its grazing on contaminated periphyton as its main food source (Neumann 1961, Skoog 1978, Walker et al. 2012). Furthermore, there is so far little knowledge about the Danubian form of *T. fluviatilis* and its ecological demands in the River Rhine, let alone its sensitivity and responses towards metal exposure. Ecotoxicological studies using *T. fluviatilis* are rare, despite its wide distribution and its key role in river ecosystems as a typical macroinvertebrate grazer (Bunje 2005, Correia et al. 2013, Zettler 2008). There are investigations in Swedish harbours about the effects of metal pollution on the brackish water form of *T. fluviatilis* (Bighiu et al. 2017a, Bighiu et al. 2017b) and a laboratory study examining effects of cadmium exposure on *T. fluviatilis* from Central Portugal (Correia et al. 2013). So far, only one ecotoxicological study has been conducted with the Danubian form of *T. fluviatilis* in particular, being a laboratory experiment testing the effects of copper exposure on the snail (Rothmeier et al. 2020). Thus, **paper 2** complements the existing fragmentary knowledge about the Danubian form of *T. fluviatilis* by a multivariate field study in the anthropogenically degraded habitat of the River Rhine, investigating the effects of metal pollution on different biological response variables of the snail.

The results of **paper 2** show that, among the measured metals, only aqueous chromium concentration is correlated to negative effects on the physiology of the Danubian form of *T. fluviatilis*, causing damage in the reproductive organs of male snails. Regarding copper concentrations, the investigations fit the results about the copper sensitivity of the snail form

derived from laboratory investigations (Rothmeier et al. 2020), indicating that the Danubian *T. fluviatilis* is able to cope with copper levels in the River Rhine. Despite from the gonad disturbance related to chromium exposure, none of the other analysed physiological, organism, and population parameters of the Danubian form of *T. fluviatilis* is negatively correlated to the measured metal concentrations. Therefore, it is assumed that the introduced snail form is little affected by evident metal pollution levels in the field. Actually, as the snail is spreading along the River Rhine and reaches partly high population densities, its ability to establish stable populations in this anthropogenic degraded habitat is indicated further (Baur et al. 2022, Gergs et al. 2015, Rothmeier and Martens 2019, Westermann et al. 2007).

The detected negative effects of chromium exposure on the grazer *T. fluviatilis* in the River Rhine (**paper 2**) must not be neglected and concentrations of this harmful metal together with its effects on aquatic biota need to be carefully monitored in the future. Though, the absence of effects of other measured metal concentrations on the Danubian form of *T. fluviatilis* raises the question about the suitability of this snail form as an indicator for metal pollution, which represents the second aim of **paper 2** (see chapter 3).

# 3 *Theodoxus fluviatilis* as an indicator organism

Early works in ecotoxicology dealt with the detection and determination of chemicals in the abiotic environment or in samples of animals and plants, which alone could neither produce meaningful statements about the effects on individual organisms, nor on populations or communities (Walker et al. 2012). There is a multitude of factors which affect responses of species to chemicals in the environment, e. g. temperature fluctuations, interactions with other pollutants, pH, salinity, the presence of mixtures, or species interactions (Walker et al. 2012). Additionally, there are large interspecific differences in the sensitivity and responses towards toxicants, which has to be considered in the assessment of pollution levels in the environment (Hoekstra et al. 1994).

There are various strategies to analyse the different responses of populations and organisms towards contaminants and to reflect their situation in the environment. Exemplary approaches are monitoring the presence or absence of (indicator) taxa from clean or polluted sites, analysing the concentrations of pollutants in indicator species collected from the field, assessing the effects of chemicals on indicator organisms regarding a wide range of biochemical and physiological parameters, or detecting the genetic resistance of species or genetic strains to toxicants (Fent 2013, Walker et al. 2012). Hence, the use of indicator organisms is a key point in ecotoxicology and essential for the assessment of environmental pollutants.

The choice of suitable indicator organisms with respect to the particular study aim is important for a reliable and meaningful assessment of pollutants in ecotoxicology and biological monitoring (Fent 2013, Hopkin 1993, Walker et al. 2012). Suitable organisms or communities function as indicators for environmental stressors due to their measurable reaction on the exposure towards certain toxic substances (Fent 2013). Gastropods are often used as indicator organisms for environmental pollution in general and metals in particular, as they possess great biomonitoring and biosensing potential (Dhiman and Pant 2021). They are an ecologically meaningful group with essential functions in freshwater ecosystems due to their role as grazers of periphyton and as prey for higher trophic levels. The removal or decline of snail populations can have significant effects on the ecosystem structure and function (Bighiu et al. 2017a, Crichton et al. 2004). Snails show several measurable responses towards metal exposure, e. g. on the physiological level the detoxification mechanisms of binding metals to

heat stock proteins and metallothioneins in their digestive gland (Amiard et al. 2006, Dhiman and Pant 2021), or the accumulation of high concentrations of metals in their tissues (Desouky 2006, Mahmoud and Abu Taleb 2013). Due to this potential, numerous ecotoxicological studies have used snails as test organisms to analyse the negative effects of metal exposure on feeding (Cain et al. 2016, Das and Khangarot 2011), reproduction (Das and Khangarot 2011, Rothmeier et al. 2020), egg development (Gao et al. 2017, Khangarot and Das 2010), locomotion (Das and Khangarot 2011, Gao et al. 2017), growth (Brix et al. 2011, Ng et al. 2011), and survival (Dhiman and Pant 2021, Gao et al. 2017, Rothmeier et al. 2020).

In this thesis, the second aim of **paper 2** is to determine if the Danubian form of the freshwater gastropod *T. fluviatilis* is a suitable indicator organism for environmental monitoring in the River Rhine, which has not yet been evaluated. The species *T. fluviatilis* has always been an important indicator for river assessment, especially within the framework of the WFD (IKSR/CIPR/ICBR 2021a, IKSR/CIPR/ICBR 2015). However, the actual situation that the native Northern-European form has been replaced by the Danubian form in the River Rhine is not yet being considered. In fact, the most recent official biological monitoring report recognises the Danubian *T. fluviatilis* as a newly introduced form of the species in the river, but suggests valuing the cryptic invader equally to the native form because of their same ecological role in the River Rhine ecosystem (IKSR/CIPR/ICBR 2021a). Consequently, this perception contributes to the assessment that the ecological status of the river has improved due to the high abundance of the Danubian *T. fluviatilis* in the River Rhine (IKSR/CIPR/ICBR 2021a). However, the evident genetic difference between the native and the introduced form of *T. fluviatilis* raises the question if treating them equally is a reliable practice for river status assessment.

The results of **paper 2** show no responses of the analysed specimens of the Danubian form of *T. fluviatilis* towards any of the analysed metals except chromium at the sampling sites. Together with the findings of partly high metal concentrations at particular sites, it is assumed that the Danubian form of *T. fluviatilis* is only restrictedly suitable as an indicator organism for at least metal pollution in the River Rhine. Hence, the role of the Danubian form of *T. fluviatilis* as an indicator organism for River Rhine assessment and the practice of using its occurrence as an argument for ecological quality improvement has to be reconsidered. This statement is supported by the findings of Bernauer (personal communication), who performed investigations on the ecological status and potential of the Middle Rhine on basis of the macroinvertebrate taxa community composition, the saprobic index, and the Potamon-Typie-

Index (PTI). The PTI is an index for the assessment of the ecological quality of large rivers within the framework of the WFD and describes the status of the macroinvertebrate communities on basis of indicator values of the river-typical taxa (Schöll and Haybach 2001). Bernauer (personal communication) analysed the PTI-based calculations of the ecological status at 41 sites at the Middle Rhine comparing results with and without considering *T. fluviatilis* as a native species. Including *T. fluviatilis* as a native species, 38 out of 41 sites showed a good ecological status. When removing *T. fluviatilis* as a native species from the calculations, 27 out of 41 sites showed a good ecological status and the results were to a smaller extent statistically validated. These findings are considered as arguments for the allocation of the Danubian form of *T. fluviatilis* in the River Rhine to the neobiotic, Ponto-Caspian species, which have a lower indicator potential than the native, Northern-European form (Bernauer, personal communication).

The cryptic invasion of a distinct haplotype of a long-established and typical indicator species in the River Rhine together with the retraction of the native haplotype into smaller tributaries is a special situation. On the ecological equality or inequality of the two forms of *T. fluviatilis* can only be speculated, as there is a lack of comparative investigations of Danubian and Northern-European specimens. Though, the intraspecific variability in *T. fluviatilis*, i. e. the phenotypic and genetic variation within the species (Cianciaruso et al. 2009), could possibly entail differences in the ecological demands and pollutant sensitivities between the phylogenetically distinct haplotypes. For example, it is already known that brackish water specimens of *T. fluviatilis* in the Baltic Sea possess a higher tolerance towards salinity changes than those from freshwater habitats (Kangas and Skoog 1978). For the Danubian form of *T. fluviatilis* in its original habitat of the Ponto-Caspian region, ecological demands that differ from those of the Northern-European form in Central Europe have already been assumed as well (Nesemann 1993), although they have not yet been verified by scientific studies. The field study conducted in **paper 3** of this thesis is a first step towards the detection of differences between the Danubian and the Northern-European form of *T. fluviatilis* and aims at analysing parasitological and physiological differences between the two forms by examining specimens from different sites histopathologically.

The results of **paper 3** show a significantly higher infection of the Northern-European form of *T. fluviatilis* with a Ponto-Caspian parasitic trematode, *Plagioporus* cf. *skrjabini*. The parasite prevalence is analysed to be the factor which mostly contributes to the differences between

the two snail forms. *P*. cf. *skrjabini* has presumably been recently co-introduced into the River Rhine system by its second intermediate or final hosts, the invasive amphipod *Dikerogammarus villosus* (Sowinsky, 1894) or Ponto-Caspian gobies like *Neogobius melanostomus* (Pallas, 1814), via the Main-Danube canal. **Paper 3** is the first record of this Ponto-Caspian parasite in native specimens of the Northern-European form of *T. fluviatilis* in the River Rhine system. It is an example of a parasitic spillover, i. e. the introduction of new parasite species through invading species together with the infection of local hosts (Emde et al. 2012, Hohenadler et al. 2019, Kelly et al. 2009), which often leads to a higher parasite virulence in the native than in the alien hosts (Lymbery et al. 2014). These findings prove evidence for a difference between the two forms of *T. fluviatilis* regarding parasitation together with physiological findings like the damage of the snail's digestive glands and pigment depositions in their stomachs. Unfortunately, a high degree of parasite infection is often accompanied by a severe damage of the infected snail's organs, which limits the comparative histopathological examination of the specimens collected from the field.

As the two forms of *T. fluviatilis* occur in different environments – the Danubian form in the River Rhine and the Northern-European form in smaller tributaries – conclusions about a possible intraspecific variability regarding ecological demands and pollutant sensitivity can so far only be assumed. As a complement to the field investigations, comparative experiments with Danubian and Northern-European specimens under standardised laboratory conditions provide the possibility to analyse intraspecific differences in *T. fluviatilis*. In **paper 4** of this thesis, a test design for a comparative laboratory experiment with the Danubian and the Northern-European form of *T. fluviatilis* is developed, conducted, and evaluated. Aim 1 of **paper 4** is to conduct the developed test design with juvenile specimens of the two different haplotypes and to examine if the snails significantly grow over the experimental time. Additionally, differences between the test animals of the two forms are examined by analysing the parameters survival, growth, activity, and organic alterations using histopathologic methods (aim 2). The results of **paper 4** show no significant differences between the Danubian and the Northern-European form of *T. fluviatilis* regarding any of the analysed parameters during the experiment without stressor substance. Hence, the suitability of the developed experimental design and the comparability of Northern-European and Danubian specimens under standardised laboratory conditions can be assumed.

# 4 Conclusion and perspectives

As metal pollution poses a major threat to aquatic ecosystems and freshwater organisms (Carpenter et al. 2011, Fent 2013, Mussali-Galante et al. 2013, Rainbow 2002, Walker et al. 2012), this thesis aims at examining the effects of metal contamination on the community, population, organism, and physiological level of biological organisation in the anthropogenic degraded environment of the River Rhine system. Based on the findings derived from the field studies in **paper 1** and **2**, the concentrations of metals in the River Rhine, especially of the metal chromium, have to be monitored closely, regarding not only aqueous, but also biofilm concentrations. To examine the effects of metal pollution on aquatic organisms, it is important not to conduct the chemical and the biological monitoring separately, but to relate metal concentrations to biological responses of communities or suitable indicator taxa. Furthermore, it has to be included in river monitoring that freshwater biota are affected by multiple abiotic and biotic stressors, including a range of highly variable effects (Lemm et al. 2021, Ormerod et al. 2010, Schäfer et al. 2016). Given the strong human pressure on the River Rhine system including different stressor types and substances, it will be a major challenge in the future to understand processes linking multiple-stressor effects to organisms, populations, and communities (Ormerod et al. 2010) and to reach the objective of a good ecological status of the River Rhine.

In the River Rhine system, the cryptic invasion of the Danubian form of the freshwater snail *T. fluviatilis* from the Ponto-Caspian region occurred, whereas the native Northern-European form showed a retraction into smaller tributaries. Actually, the native form has simply been replaced by the Danubian cryptic invader as an indicator organism in the practice of biological River Rhine monitoring, though the equal treatment of the two forms is questionable. In **paper 2** of this thesis, it is shown that the Danubian form of *T. fluviatilis* is only restrictedly suitable as an indicator organism for metal pollution in the River Rhine. **Paper 3** demonstrates differences between the two forms of the species in the field, regarding parasitation and pathologic physiological findings. An intraspecific variability between the Northern-European and the Danubian form of *T. fluviatilis* is possible and has to be further investigated, e. g. in laboratory experiments comparing the snail forms with regard to their sensitivity towards pollutants. In **paper 4** of this thesis, a laboratory test design for a growth experiment with the

two forms is developed and evaluated. On this basis, the next step to detect intraspecific differences would be complementing the experiment by adding a stressor substance, e. g. a metal, and comparing the sensitivities between the different snail forms. If an intraspecific variability regarding pollutant sensitivity between the Northern-European and the Danubian form of *T. fluviatilis* can be verified, an important argument is provided for the assumption that the cryptic invader cannot be regarded as equal to the native form of the species and is no suitable indicator for environmental monitoring in the River Rhine.

In general, intraspecific variability towards contaminants is poorly recognised in ecotoxicological studies, though it poses a potential source of error in interpreting the results of laboratory and field investigations (Dumont et al. 2019, Petitjean et al. 2021). Sensitivity towards pollutants can vary greatly within a species and intraspecific variability of e. g. metal sensitivity has already been observed for aquatic macrophytes (Dumont et al. 2019), *Daphnia* (Barata et al. 2002), gudgeon fish (Petitjean et al. 2021), and the freshwater snail *Lymnaea stagnalis* (Linné, 1758) (Côte et al. 2015). Thus, conclusions about the effects of contaminants on organisms can only be safely drawn if the respective population or genetic lineage of the examined test species is considered (Côte et al. 2015, Petitjean et al. 2021). To better predict and evaluate the effects of environmental stressors on aquatic organisms, possible intraspecific variabilities have to be considered not only in laboratory studies, but also in the environmental monitoring and assessment of freshwater ecosystems.

Pollutant sensitivity cannot only vary within a species, but also between different life cycle stages of a test species (Preston and Snell 2001). Growth experiments with juvenile test organisms are important to analyse the effects of pollutants on freshwater organisms, as developing life stages are highly vulnerable and sensitive to contaminants (Khangarot and Das 2010, Langston et al. 1998). Though, for a holistic investigation of the effects of environmental pollution, the importance of full life-cycle toxicity assessment is emphasised. As organisms in natural ecosystems are exposed to contaminants at different stages during their life cycle, examining the sensitivity of all life stages of test organisms would ensure that the most sensitive stage of an organism is recognised (Preston and Snell 2001). The developed laboratory test design in **paper 4** can possibly be further extended and conducted with different life stages of *T. fluviatilis*.

The most apparent difference between the spatially separated populations of the Northern-European and the Danubian form of *T. fluviatilis* in the River Rhine system is so far, besides the visual differences in size and shell pattern, the higher parasitation of native snail specimens by the trematode *P*. cf. *skrjabini* (**paper 3**). Organisms in freshwater environments are commonly parasitised and therefore simultaneously confronted with infections and adverse environmental conditions. Contaminants and parasitism are two stressors that can interact in different ways, possibly resulting in stronger impacts of the parasite, the toxicant, or both (Bighiu et al. 2017b, Coors et al. 2008, Sures 2008). Several studies highlight the importance of investigating the combined effects of parasitism and pollution to examine the health and condition of host populations (Bighiu et al. 2017b, Kelly et al. 2010, Sures 2008, Sures et al. 2017). In light of the decline of the native Northern-European form of *T. fluviatilis* in the anthropogenically influenced River Rhine system and its high parasitation, the conditions of this snail form regarding both infection and pollution, as well as interactions of these factors, need to be subject of future studies. If the health of the remaining populations of the Northern-European form of *T. fluviatilis* in smaller tributaries of the River Rhine system is seriously threatened by parasite pressure together with environmental pollution, a further decline of the native snail form can be hypothesised, which would result in the loss of biological diversity.

The example of the invasion of a non-native haplotype of *T. fluviatilis* in the River Rhine system provides the possibility to improve our knowledge about cryptic invasions, a field which is largely neglected and forms a minor part of current research on biological invasions (Morais and Reichard 2018). Cryptic invasions are not well documented, though they have the potential to affect native communities, as cryptic invaders may replace native lineages, take new roles in biological interactions, and possibly affect community structure and ecosystem functioning, together with the loss of geographic variation (Morais and Reichard 2018). Hence, the populations of the freshwater snail *T. fluviatilis* in the River Rhine system provide the possibility to complete the current fragmentary knowledge of cryptic invasions and their consequences.

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## Papers

**Paper 1**

Is metal pollution a relevant environmental factor affecting the macroinvertebrate community in the River Rhine system?

**Paper 2**

The Danubian cryptic invader *Theodoxus fluviatilis* (Gastropoda: Neritidae) in the River Rhine: a potential indicator for metal pollution?

**Paper 3**

The Ponto-Caspian parasite *Plagioporus* cf. *skrjabini* reaches the River Rhine system in Central Europe: higher infestation in the native than in the introduced Danubian form of the gastropod *Theodoxus fluviatilis*

**Paper 4**

Evaluation of a preliminary test design for a growth experiment with juveniles of two phylogenetic different forms of the freshwater snail *Theodoxus fluviatilis*

# Paper 1

# Is metal pollution a relevant environmental factor affecting the macroinvertebrate community in the River Rhine system?

Louisa Marie Rothmeier[1], Andreas Martens[2], Karsten Grabow[3], Jennifer Bartz[4], René Sahm[5]

**Abstract** Metal pollution is a relevant threat for biodiversity in freshwater ecosystems, especially in anthropogenically degraded large rivers and their catchment areas. The uptake and accumulation of metals by aquatic macroinvertebrates can have effects on the individual, community, and ecosystem level. Simultaneously, anthropogenic activities lead to a multitude of impairments for river ecosystems besides metal pollution, like inter alia nutrient input and the occurrence of non-native species. Given the importance of metal pollution for aquatic biota, we hypothesise that this stressor is one of the most important driving factors for structuring the macroinvertebrate community in the River Rhine system. We performed a field sampling at 30 sites at the Upper River Rhine and three tributaries, analysing relationships between 25 environmental parameters, including metal concentrations in water and biofilms, and the macroinvertebrate community. Our results show that the geogenic originated substances calcium, sulphate, and strontium were of a higher relevance for the macroinvertebrate community composition than the metals copper, chromium, zinc, and iron. In contrast to our hypothesis, metals do not seem to be the most important driving factor for structuring the macroinvertebrate community with regard to the analysed metals and taxa at the sampling sites in our study. Further, we found a clear separation of the macroinvertebrate community structure between the River Rhine and the tributaries, with a dominance of invasive species only in the River Rhine itself. Given the significant difference between the communities of the River Rhine and the tributaries, we assume that the occurrence of invasive species is a factor potentially masking the effects of other stressors with smaller impacts, e. g. metal pollution, in our study. As multiple stressors are common in stream and river ecosystems, we emphasise the importance of analysing both abiotic and biological pressures and their interactions to understand their impacts.

**Keywords** Anthropogenic degradation, Non-native species, Tributaries, Geogenic originated factors, Multiple stressors

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Running title: Impact of metal pollution on macroinvertebrates in the River Rhine system

## Introduction

Freshwater ecosystems are well known as hotspots of biodiversity, and especially large rivers are the most important source of renewable water supply for humans and ecosystems (Vörösmarty et al. 2010). Simultaneously, rivers are among the most degraded ecosystems worldwide due to a long history of intensive human pressures, which pose a threat to their function and ecological integrity (Urbanič et al. 2021). Multiple potential stressors are regarded to be commonly occurring in stream and river ecosystems (Lemm et al. 2020, Schäfer et al. 2016), whereby key issues of anthropogenically influenced ecological impairment comprise habitat degradation, pollution, water resource development, and biotic factors (Schäfer et al. 2016, Vörösmarty et al. 2010). As pressures are various, the knowledge of the importance of specific environmental factors in field studies is mandatory for river management (Ormerod et al. 2010, Townsend et al. 2008). Here, special challenges are faced in cases where multiple factors interact and mask possible effects of each other (Liess & Von der Ohe 2005, Ormerod et al. 2010).

Among all anthropogenic stressors, metal pollution is still an actual threat especially in large rivers. It often causes negative effects on the individual fitness of freshwater organisms, leading to consequences for the population and community levels and therefor potentially also for the integrity of the whole ecosystem (Mussali-Galante et al. 2013, Tovar-Sánchez et al. 2018). Metals like copper, zinc, or chromium are well known to be toxic to biota, able to bioaccumulate in biofilms or living organisms, and are persistent in the ecosystem for long periods of time (Hoffmann & Willi 2008, Mussali-Galante et al. 2013, Rothmeier et al. 2020, Valavanidis & Vlachogianni 2010). Given the high relevance of metal pollution for biota in freshwater ecosystems together with the co-occurrence and interactions of a multitude of other stressors, scientific studies considering metal pollution together with other potential stressors are necessary for a more holistic understanding of the effects on organisms and ecosystems (Lemm et al. 2020, Ormerod et al. 2010, Townsend et al. 2008).

The River Rhine is one of the most anthropogenically degraded river ecosystems in Central Europe because of its huge importance for shipping and connected industrial locations along with an irreversible habitat modification also by hydraulic engineering (Leuven et al. 2009). In the context of the Water Framework Directive (WFD; 2000/60/EC), which was adopted in the year 2000 to achieve a “good status” of the natural surface waters or a “good potential” for heavily modified water bodies of all European Union member states (European Community 2000), the water quality of the River Rhine is monitored regularly with regard to the chemical and biological quality. Though the overall chemical water quality is improving, and most pollutants measured within the official monitoring program do not exceed the environmental quality standards in general, concentrations of inter alia copper, cadmium, and zinc might be still problematic for aquatic biota at least on a lower spatial scale (IKSR/CIPR/ICBR 2021b). Furthermore, concentrations of pollutants in biofilms or sediments are not considered by the routine monitoring program, which can lead to underestimations given the high bioaccumulation potential of metals (Morin et al. 2008, Mussali-Galante et al. 2013).

The ecosystem of the River Rhine is not only influenced by abiotic contaminants, but also by biological impairments, e. g. invasive species, which are generally regarded as one of the major threats to the biodiversity of freshwater ecosystems (Chandra & Gerhardt 2008, Richardson & Ricciardi 2013, Vitousek et al. 1997). Along with a general dramatic increase of invasive species in the rivers of Central Europe through the

development of a Eurasian network of waterways and the removal of barriers in potential invasion corridors (Arndt et al. 2009, Bij de Vaate et al. 2002, Leuven et al. 2009), the benthic community in the River Rhine system is nowadays affected by the occurrence of many non-native macroinvertebrate taxa (Arndt et al. 2009, Leuven et al. 2009). As the macroinvertebrate community of rivers reflects the sum of all influencing or disturbing factors, the impairments of the decline of native species and the domination of ubiquitous taxa through the introduction of invasive species (IKSR/CIPR/ICBR 2021a, Leuven et al. 2009) occur simultaneously with changes in the community composition, richness and species diversity through the exposure to abiotic stressors (Arndt et al. 2009, Mussali-Galante et al. 2013, Rainbow 2002, Szöcs et al. 2012).

Based on the knowledge about the biological degradation through anthropogenic influenced stressors, we hypothesise that metal pollution is one of the most important driving factors for structuring the macroinvertebrate community in the River Rhine system. We conducted a field investigation at sampling sites in the River Rhine and three of its tributaries to examine if metal concentrations in water and biofilms are significantly correlated to the identified macroinvertebrate taxa in light of possible other influences through nutrient concentrations, geogenic originated factors or the occurrence of non-native species.

## Methods

*Study sites and measured environmental parameters*

Sampling was conducted at 30 sites (S1 - S30, Figure 1) of the River Rhine system (main river and three large tributaries) from August to September in 2018 and 2019. A total of 23 sites were located at the River Rhine, covering 116 kilometres from river-km 316 to river-km 432 (S1 - S23), one site was located at the river Jagst (S24), three sites at the river Kocher (S25 - S27), and three sites at the river Tauber (S28 - S30). The sites were selected to be heterogeneous with regard to apparent anthropogenic influences in order to be presumably different in their metal and nutrient exposure, resulting in sites close to tributaries, ferry ports, or within industrial harbours and marinas of the River Rhine as well as sites with less anthropogenic influence at the smaller rivers Jagst, Kocher, and Tauber. Sites were chosen to be comparable regarding visual environmental characteristics (i. e. substrate and the occurrence of aquatic macrophytes) to minimise the potential impact of these factors (for site details see Table S1).

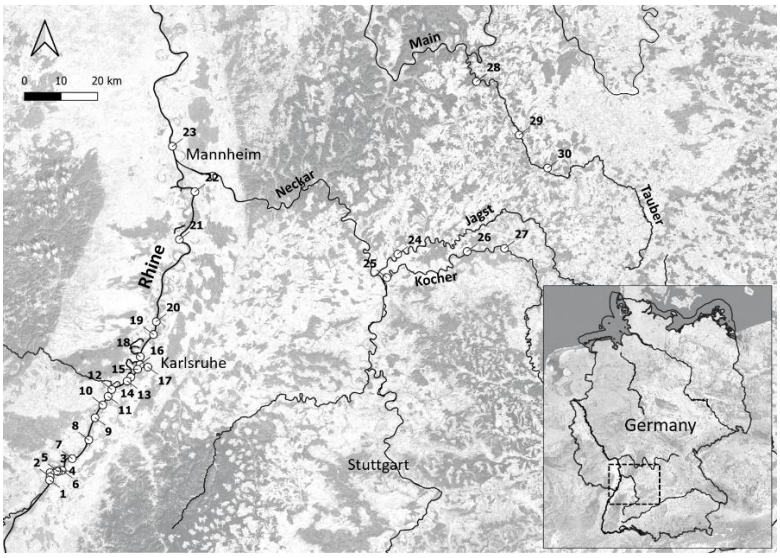


**Figure 1** Study area at the German River Rhine system. Map showing the bigger 1st order rivers in overview (large-scale map) and the 30 sampling sites at the Upper River Rhine and the rivers Jagst, Kocher and Tauber (detail map). Sampling locations are marked by dots with the respective site numbers (for coordinates and site details see Table S1). Map created with the program QGIS (QGIS Development Team 2020).

At each sampling site, 25 environmental parameters were measured (Table 1). Water temperature, pH, conductivity, and dissolved oxygen were measured directly in the field (using DO-100, PH-100 ATC, LWT-01, and DO-100, respectively; Voltcraft, Switzerland). Water samples (1 L) were transported to the laboratory in polypropylene bottles and a subsample of 250 ml was frozen at -22 °C for nutrient measurements until further processing. Aqueous concentrations of chloride [$Cl^-$], nitrite [$NO_2^-$-N], nitrate [$NO_3^-$-N], phosphate [$PO_4^{3-}$-P], sulphate [$SO_4^{2-}$], ammonium [$NH_4^+$-N], calcium [$Ca^{2+}$], magnesium [$Mg^{2+}$], sodium [$Na^+$], and silicium [Si] were analysed from 0.45 µm filtered water samples using continuous-flow-analysis (SAN++, Skalar, The Netherlands) and ion chromatography (Metrohm, Switzerland). For metal analyses, water subsamples (total volume 40 ml) were filtered (0.45 µm) and biofilm subsamples were scraped from a defined stone surface with a spatula and stored in river water (total volume 10 ml). Both water and biofilm subsamples for metal analyses were acidified with ultrapure nitric acid (Merck, Germany) to a final concentration of 0.5% until further analysis. Water and biofilm concentrations of the five metals copper [Cu], chromium [Cr], zinc [Zn], strontium [Sr], and iron [Fe] were analysed using inductively coupled plasma optical emission spectrometry (Perkin Elmer, USA; for details on the conducted methods regarding nutrient and metal measurements see also Rothmeier et al. 2020). Periphyton biomass subsamples (five samples per site) were filtered on precombusted glass-fibre filters (Whatman GF6, Ø 25 mm, Maidstone, UK) for ash-free dry mass (AFDM) and dried at 105 °C for 24 h. After weighing (dry mass), the filters were combusted at 550 °C for 8 h and weighed again. The AFDM was calculated by subtraction.

**Table 1** Measurement ranges of the 25 environmental parameters at 30 sampling sites at the German Upper River Rhine and the tributary rivers Jagst, Kocher, and Tauber. For exact measurement values at each site see Table S2.

| Compartment | Environmental variable | Unit | Range |
|---|---|---|---|
| Water | Temperature | °C | 15.4 - 26.1 |
| | pH | - | 7.7 - 9.0 |
| | Conductivity | µS $cm^{-1}$ | 230 - 856 |
| | Dissolved oxygen | mg $L^{-1}$ | 6.0 - 10.4 |
| | Chloride ($Cl^-$) | mg $L^{-1}$ | 12 - 66 |
| | Nitrite ($NO_2^-$-N) | mg $L^{-1}$ | 0.002 - 0.030 |
| | Nitrate ($NO_3^-$-N) | mg $L^{-1}$ | 0.4 - 6.3 |
| | Phosphate ($PO_4^{3-}$-P) | mg $L^{-1}$ | 0.001 - 0.075 |
| | Sulphate ($SO_4^{2-}$) | mg $L^{-1}$ | 21 - 200 |
| | Ammonium ($NH_4^+$-N) | mg $L^{-1}$ | 0.006 - 0.030 |
| | Calcium ($Ca^{2+}$) | mg $L^{-1}$ | 25 - 200 |
| | Magnesium ($Mg^{2+}$) | mg $L^{-1}$ | 6.2 - 20.0 |
| | Sodium ($Na^+$) | mg $L^{-1}$ | 5.0 – 46.2 |
| | Silicium (Si) | mg $L^{-1}$ | 0.3 - 3.8 |
| | Copper (Cu) | µg $L^{-1}$ | 1.2 - 9.5 |
| | Chromium (Cr) | µg $L^{-1}$ | 0.8 - 3.6 |
| | Strontium (Sr) | µg $L^{-1}$ | 213 - 2,000 |

| | | | |
|---|---|---|---|
| | Zinc (Zn) | µg $L^{-1}$ | 2.1 - 17.6 |
| | Iron (Fe) | µg $L^{-1}$ | 34 - 455 |
| Biofilm | Copper (Cu) | mg $kg^{-1}$ | 5.4 - 28.6 |
| | Chromium (Cr) | mg $kg^{-1}$ | 7.4 - 40.8 |
| | Strontium (Sr) | mg $kg^{-1}$ | 96 - 969 |
| | Zinc (Zn) | mg $kg^{-1}$ | 31 - 155 |
| | Iron (Fe) | mg $kg^{-1}$ | 4,820 – 14,260 |
| | Biomass of periphyton (AFDM) | mg $cm^{-2}$ | 0.2 - 3.7 |

*Sampling of macroinvertebrate taxa*

At each site, macroinvertebrates were brushed from ten randomly collected riprap stones, which were wrapped in aluminium foil to estimate sampling surface: by weighing reference aluminium foil pieces, the function of foil weight to corresponding surface value was calculated. Resulting from the sampling surface per site, the density of each identified taxon (ind $m^{-2}$) was calculated (see also Rothmeier et al. 2022). Animals were collected with a sieve (200 µm mesh size), stored in 96% ethanol, and transported to the laboratory for identification of taxa. All invertebrates were counted and identified using an EZ4 microscope (8x magnification, Leica, Germany) to the lowest practical taxonomic level, which was by default species level, if possible, as suggested by Jones (2008). For the freshwater snail *Theodoxus fluviatilis* (Linnaeus, 1758), we distinguished between the native Northern-European and the newly introduced Danubian form, which differ morphologically (Gergs et al. 2015).

*Data analysis*

The concentrations of metals in water samples lower than the limit of quantification (LOQ) were set as LOQ/2 (Clarke 1998), and those lower than the limit of detection (LOD) were taken as zero values (i.e., LOQ/LOD: Cu 2.2/0.7 µg/L, Cr 0.7/0.2 µg/L, Sr 0.02/0.01 µg/L, Zn 1.1/0.3 µg/L, Fe 0.8/0.2 µg/L). All calculations and statistical analyses were conducted with the program R (version 4.1.2, R Core Team 2021). Environmental (Table 1) and macroinvertebrate taxa density data (Table 2) were standardised to zero mean and unit variance for dimensionally heterogeneous variables. The environmental variables were log(x) transformed prior to analysis to improve their distribution. The densities of macroinvertebrate taxa were Hellinger-transformed to give low weights to taxa with low densities and many zeros (Legendre & Gallagher 2001).

To examine the effects of the environmental variables on the macroinvertebrate taxa densities, a distance-based redundancy analysis (db-RDA) was used, a constrained ordination method which allows to calculate a dissimilarity matrix of every distance measure (Legendre & Anderson 1999). Since there were taxa densities of zero, Bray-Curtis dissimilarity was used as distance measure. The environmental variables' variance inflation factors (VIFs) were computed, as strong linear dependencies (auto-correlations) are possible in a large set of explanatory variables. As 23 out of 25 environmental variables showed VIFs > 10, which indicates strong collinearity (Borcard et al. 2018), a forward selection of the variables was conducted: The function *ordiR2step()* of the R package vegan (version 2.5-7, Oksanen et al. 2020) was used to find a parsimonious model with the most influential environmental variables using two stopping criteria: permutation p-values (999 permutations) and the adjusted $R^2$ of the global model ($R^2global_{adj}$ = 0.59; Borcard et al. 2018).

The significance of the db-RDA results was analysed by permutation tests using the function *anova()* of the R package vegan (999 permutations). To determine the significance of the relationships between single taxa densities and measurements of the selected environmental variables, Generalised Linear Models (GLM, error distribution = Gaussian) were conducted. The number of significant ($\alpha = 0.05$) positive or negative correlations between the selected environmental variables and the taxa densities was derived from the GLM analysis as a measure for the importance of the relevant environmental variables for the macroinvertebrate taxa.

For the analysis of the macroinvertebrate community and to compare the density data of the macroinvertebrate taxa between the River Rhine and the tributaries Jagst, Kocher, and Tauber, a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted using the function *adonis()* of the R package vegan (version 2.5-7, Oksanen et al. 2020), with Bray-Curtis dissimilarity as distance measure (999 permutations) and sampling sites as replicates. Here, to analyse the percentual contribution of the single taxa to the dissimilarity between the sites at the River Rhine and at the tributaries, the taxa's similarity percentages were calculated using vegan's function *simper()*.

## Results

### *General description of the dataset*

A total of 25 macroinvertebrate taxa were identified at the 30 study sites, 18 of them native and seven non-native taxa (Table 2, Table S2). The non-native taxa recorded were the Danubian form of *T. fluviatilis*, *Potamopyrgus antipodarum* (Gray, 1843), *Physella acuta* (Draparnaud, 1805), *Dreissena* spp. (*D. polymorpha* (Pallas, 1771) and *D. rostriformis* (Deshayes, 1838)), *Jaera sarsi* Valkanov, 1936, *Dikerogammarus villosus* (Sowinsky, 1894), and *Chelicorophium* spp. (Table 2). The taxa which exclusively occurred in the sampled tributaries of the River Rhine were Hirudinea, the native Northern-European form of *T. fluviatilis*, *Asellus aquaticus* (Linnaeus, 1758), Gammaridae, *Leuctra* sp., *Calopteryx splendens* (Harris, 1782), *Aphelocheirus aestivalis* (Fabricius, 1794) and Coleoptera (Table S2). Taxa that were only found in the River Rhine were all non-native taxa, with exception of the New Zealand mud snail *P. antipodarum*, which was found in the River Rhine and in all three tributaries (Table S2).

**Table 2** Density ranges of the 25 macroinvertebrate taxa identified from 30 sampling sites at the German Upper Rhine and the tributary rivers Jagst, Kocher, and Tauber. The taxa are divided into native (first section) and non-native (second section) taxa. For exact density values at each site see Table S2.

| Taxon | Unit | Range |
|---|---|---|
| Turbellaria | ind $m^{-2}$ | 0 - 138 |
| Hirudinea | | 0 - 31 |
| Sphaeriidae | | 0 - 20 |
| Planorbidae | | 0 - 14 |
| *Bithynia tentaculata* | | 0 - 692 |
| *Radix auricularia* | | 0 - 32 |
| *Theodoxus fluviatilis* - native form | | 0 - 539 |
| *Cypridopsis vidua* | | 0 - 5 |
| *Asellus aquaticus* | | 0 - 5 |
| Gammaridae | | 0 - 39 |
| Hydracarina | | 0 - 100 |
| Ephemeroptera | | 0 - 200 |
| *Leuctra* sp. | | 0 - 348 |
| Trichoptera | | 0 - 321 |
| *Calopteryx splendens* | | 0 - 8 |
| *Aphelocheirus aestivalis* | | 0 - 5 |
| Coleoptera | | 0 - 323 |
| Diptera | | 0 - 1052 |
| *Theodoxus fluviatilis* - Danubian form | ind $m^{-2}$ | 0 - 546 |
| *Potamopyrgus antipodarum* | | 0 - 702 |

| | |
|---|---|
| *Physella acuta* | 0 - 3 |
| *Dreissena* spp. | 0 - 1017 |
| *Jaera sarsi* | 0 - 221 |
| *Dikerogammarus villosus* | 0 - 980 |
| *Chelicorophium* spp. | 0 - 112 |

The concentrations of the analysed metals were above the level of detection (LOD) at all sampling sites in water and biofilm samples (Table 1). The highest measured concentrations of copper, chromium, zinc, and iron in the biofilm and the aqueous phase were present in the River Rhine, and the highest values of aqueous and biofilm concentrations of strontium were present in the tributary river Tauber (Table S2). The highest measured aqueous concentrations of the metals chromium, zinc, and copper at our sampling sites (chromium: 3.6 µg $L^{-1}$, zinc: 17.6 µg $L^{-1}$, copper: 9.5 µg $L^{-1}$, Table 1, Table S2) were higher than the highest measurements of these metals in the actual River Rhine water quality report (chromium: 0.25 µg $L^{-1}$, zinc: 6.1 µg $L^{-1}$, copper: 2.3 µg $L^{-1}$, IKSR/CIPR/ICBR 2021b).

*Correlation between the macroinvertebrate taxa densities and the environmental variables*

Of the 25 analysed environmental variables, the most influential parameters correlating with the macroinvertebrate taxa densities were the aqueous concentrations of strontium, chromium, nitrite, sulphate, and calcium (*ordiR2step()* forward selection). The parsimonious db-RDA model of the macroinvertebrate density data constrained by these five environmental parameters revealed a significance of the global canonical relationship, without collinearity of variables ($p_{model}$ =0.001, $R^2_{adj}$ = 0.51, Figure 2). Of the five selected environmental variables, the concentrations of the metals strontium and chromium showed a significant relationship to the macroinvertebrate taxa density data (p = 0.001, Table 3a). The first two axes of the db-RDA correlation plot represent two separate groups of influential environmental variables: the x-axis explaining most of the variance of the dataset (41.5%) correlates to the influence of the geogenic originated substances calcium, sulphate, and strontium, whereby the y-axis explaining 11.5% of the variance correlates to the influence of the pollutants with anthropogenic origin (i.e. chromium and nitrite) (Figure 2).

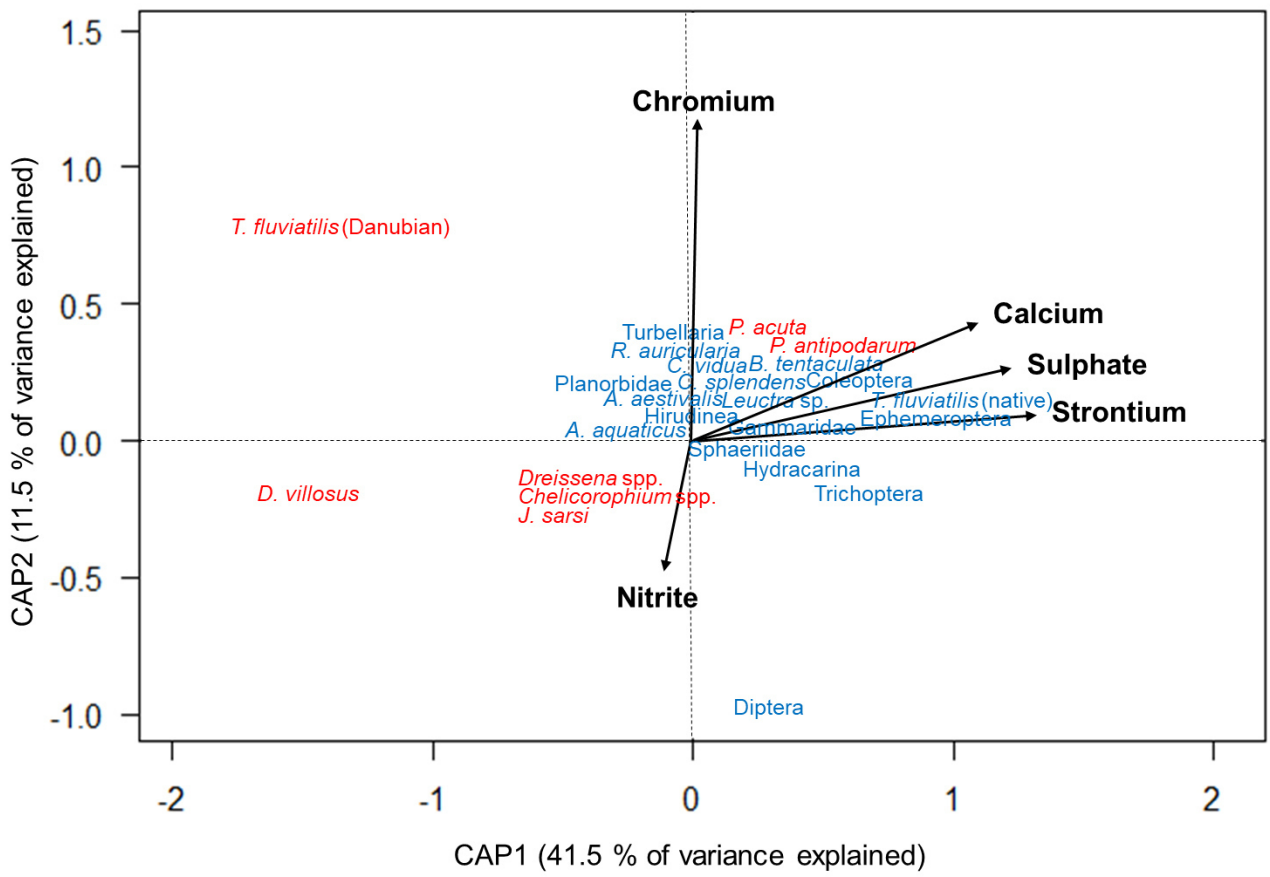


**Figure 2** Distance-based redundancy analysis (db-RDA) correlation biplot showing the first two canonical axes of the densities of 25 macroinvertebrate taxa (Table 2) constrained by the *ordiR2step()* forward selected explanatory environmental variables strontium, chromium, nitrite, sulphate, and calcium (aqueous concentrations, Table 1). The different colours of the macroinvertebrate taxa show which taxa are native (blue) and non-native (red) to the study area at the Upper River Rhine system. Scaling 2 correlation biplot, site scores (n = 30) are not displayed for clarity. Ordination based on Bray-Curtis dissimilarity and Hellinger-transformed macroinvertebrate density data.

The univariate GLM analysis revealed significant relationships between one or more of the five selected variables and the densities of 21 out of the 25 taxa (Table 3b). The differentiation of the GLM results between the densities of native and non-native taxa revealed differences in the number of significant correlations (Table 4). The metal strontium was significantly correlated to 13 out of 18 native taxa, whereas the non-native taxa *T. fluviatilis* (Danubian form) and *D. villosus* were significantly negatively correlated. Sulphate and calcium were significantly correlated to 15 out of 18 native taxa, respectively, but only to one non-native taxon, which was *P. antipodarum*. The non-native taxa *T. fluviatilis* (Danubian form), *D. villosus* and *J. sarsi* were significantly negatively correlated to sulphate and calcium. The metal chromium had a significant relationship to the macroinvertebrate community in general, but only the native taxon Diptera was significantly negatively correlated. For nitrite, the native taxa Turbellaria and Sphaeriidae showed significant correlations (Table 3 and Table 4).

**Table 3 a** Results of the permutation tests (999 permutations) of the parsimonious distance-based RDA model after the *ordiR2step()* forward selection of the five explanatory variables strontium, chromium, nitrite, sulphate, and calcium (aqueous concentrations) constraining the density data of the 25 macroinvertebrate taxa. **b** Results of the Generalised Linear Model analysis showing relationships between the selected explanatory variables and single taxa densities. The taxa are divided into native (first section) and non-native (second section) taxa. Bold values indicate significant effects ($p < 0.05$).

| Selected environmental variables | **Strontium** | | **Chromium** | | **Nitrite** | | **Sulphate** | | **Calcium** | |
|---|---|---|---|---|---|---|---|---|---|---|
| **a Distance-based RDA** | | | | | | | | | | |
| Anova (999 permutations) | F | p | F | p | F | p | F | p | F | p |
| | 23.4 | **0.001** | 6.2 | **0.001** | 2.2 | 0.061 | 1.8 | 0.095 | 1.7 | 0.131 |
| **b Generalised Linear Model** | | | | | | | | | | |
| taxon | coeff | p | coeff | p | coeff | p | coeff | p | coeff | p |
| Turbellaria | 0.02 | **< 0.05** | 4.16 | 0.41 | 2934 | **< 0.001** | 0.13 | **< 0.05** | 0.14 | **< 0.05** |
| Hirudinea | 0.005 | **< 0.05** | -0.05 | 0.97 | -337 | 0.15 | 0.05 | **< 0.01** | 0.05 | **< 0.01** |
| Sphaeriidae | 0.006 | **< 0.001** | 0.28 | 0.78 | 443 | **< 0.01** | 0.04 | **< 0.001** | 0.04 | **< 0.001** |
| Planorbidae | 0.001 | 0.35 | -0.08 | 0.88 | -101 | 0.27 | 0.01 | 0.07 | 0.01 | 0.07 |
| *Bithynia tentaculata* | 0.04 | 0.27 | 5.7 | 0.82 | -6509 | 0.13 | 0.65 | **< 0.05** | 0.67 | **< 0.05** |
| *Radix auricularia* | 0.005 | **< 0.01** | 0.22 | 0.86 | -13.8 | 0.95 | 0.037 | **0.01** | 0.04 | **< 0.05** |
| *Theodoxus fluviatilis* (native) | 0.07 | **< 0.05** | -0.66 | 0.97 | -2243 | 0.53 | 0.69 | **< 0.01** | 0.73 | **< 0.01** |
| *Cypridopsis vidua* | 0.001 | **< 0.01** | 0.15 | 0.52 | -32 | 0.42 | 0.01 | **< 0.001** | 0.01 | **< 0.001** |
| *Asellus aquaticus* | 0.0005 | 0.12 | 0.09 | 0.67 | 13.6 | 0.7 | 0.006 | **< 0.01** | 0.007 | **< 0.05** |
| Gammaridae | 0.01 | **< 0.001** | 1.16 | 0.62 | 460 | 0.25 | 0.12 | **< 0.001** | 0.12 | **< 0.001** |
| Hydracarina | 0.03 | **< 0.001** | -0.51 | 0.92 | -579 | 0.51 | 0.23 | **< 0.001** | 0.24 | **< 0.001** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Ephemeroptera | 0.06 | **< 0.001** | -2.4 | 0.81 | -1601 | 0.34 | 0.42 | **< 0.001** | 0.45 | **< 0.001** |
| *Leuctra* sp. | 0.05 | **< 0.05** | -0.94 | 0.94 | 69 | 0.98 | 0.29 | 0.07 | 0.31 | 0.06 |
| Trichoptera | 0.07 | **< 0.001** | -6.1 | 0.64 | 498 | 0.82 | 0.49 | **0.001** | 0.52 | **< 0.01** |
| *Calopteryx splendens* | 0.001 | 0.07 | -0.0004 | 0.99 | -61 | 0.27 | 0.01 | **0.01** | 0.01 | **< 0.05** |
| *Aphelocheirus aestivalis* | 0.001 | **< 0.05** | 0.002 | 0.99 | -69 | 0.05 | 0.007 | **< 0.01** | 0.008 | **< 0.01** |
| Coleoptera | 0.05 | **< 0.05** | 2.1 | 0.9 | -3063 | 0.14 | 0.47 | **< 0.001** | 0.49 | **< 0.01** |
| Diptera | 0.12 | 0.16 | -103 | **< 0.05** | 9792 | 0.24 | 0.57 | 0.36 | 0.51 | 0.44 |
| *Theodoxus fluviatilis* (Danubian) | -0.08 | **< 0.05** | 24.1 | 0.3 | -3072 | 0.44 | -0.61 | **< 0.05** | -0.64 | **< 0.05** |
| *Potamopyrgus antipodarum* | 0.04 | 0.27 | 4.87 | 0.85 | -7156 | 0.1 | 0.64 | **< 0.05** | 0.66 | **< 0.05** |
| *Physella acuta* | -0.0002 | 0.38 | 0.04 | 0.74 | -10.8 | 0.61 | -0.001 | 0.48 | -0.001 | 0.41 |
| *Dreissena* spp. | -0.04 | 0.48 | -35 | 0.36 | -504 | 0.94 | -0.39 | 0.41 | -0.46 | 0.36 |
| *Jaera sarsi* | -0.03 | 0.05 | -15.8 | 0.1 | -470 | 0.78 | -0.24 | **< 0.05** | -0.28 | **< 0.05** |
| *Dikerogammarus villosus* | -0.16 | **< 0.05** | -25 | 0.58 | -3351 | 0.67 | -1.2 | **< 0.05** | -1.41 | **< 0.05** |
| *Chelicorophium* spp. | -0.01 | 0.19 | -9.23 | 0.06 | 85 | 0.92 | -0.09 | 0.14 | -0.11 | 0.11 |

**Table 4** The five most influential environmental variables (*ordiR2step()* forward selection) constraining the densities of the 25 macroinvertebrate taxa and the number of taxa to which the parameters are significantly or significantly negative correlated ($p < 0.05$). The number of correlated taxa is differentiated between native and non-native taxa.

| | Number of taxa correlated | | Number of taxa negatively correlated | |
|---|---|---|---|---|
| Environmental variables | native taxa | non-native taxa | native taxa | non-native taxa |
| Strontium | 13 | 0 | 0 | 2 |
| Chromium | 0 | 0 | 1 | 0 |
| Nitrite | 2 | 0 | 0 | 0 |
| Sulphate | 15 | 1 | 0 | 3 |
| Calcium | 15 | 1 | 0 | 3 |

*Analysis of the macroinvertebrate community*

The results of the PERMANOVA analysis showed a significant difference between the taxa densities of the sampling sites at the River Rhine and at the tributaries (PERMANOVA, $p = 0.001$), which also represents the almost total separation between the different communities of native taxa at the tributaries and non-native taxa at the River Rhine (see Figure 3 for the biplot of the dissimilarity matrix based on Bray-Curtis-dissimilarity). Furthermore, the non-native taxa showed a subdivision into their original distribution range: first, the Ponto-Caspian taxa *Dreissena* spp., *D. villosus*, *J. sarsi*, *Chelicorophium* spec. and the Danubian form of *T. fluviatilis* were only present in and therefore showed a correlation to the River Rhine – the same was true for the North-American acute bladder snail *P. acuta*, though this taxon showed a less strong correlation to sampling sites at the River Rhine than the Ponto-Caspian taxa (Figure 3). Second, the New Zealand mud snail *P. antipodarum* was more closely correlated to the tributaries (Figure 3). Regarding the percentual contribution of single taxa to the overall dissimilarity between the sites at the River Rhine and at the tributaries (*simper()* analysis), the highest contribution showed the order Diptera (18.2%) and the species *D. villosus* (14.7%), followed by the Danubian form of *T. fluviatilis* (8.2%), the order Ephemeroptera and the native form of *T. fluviatilis* (both 6.1%), and the order Trichoptera (5.1%).

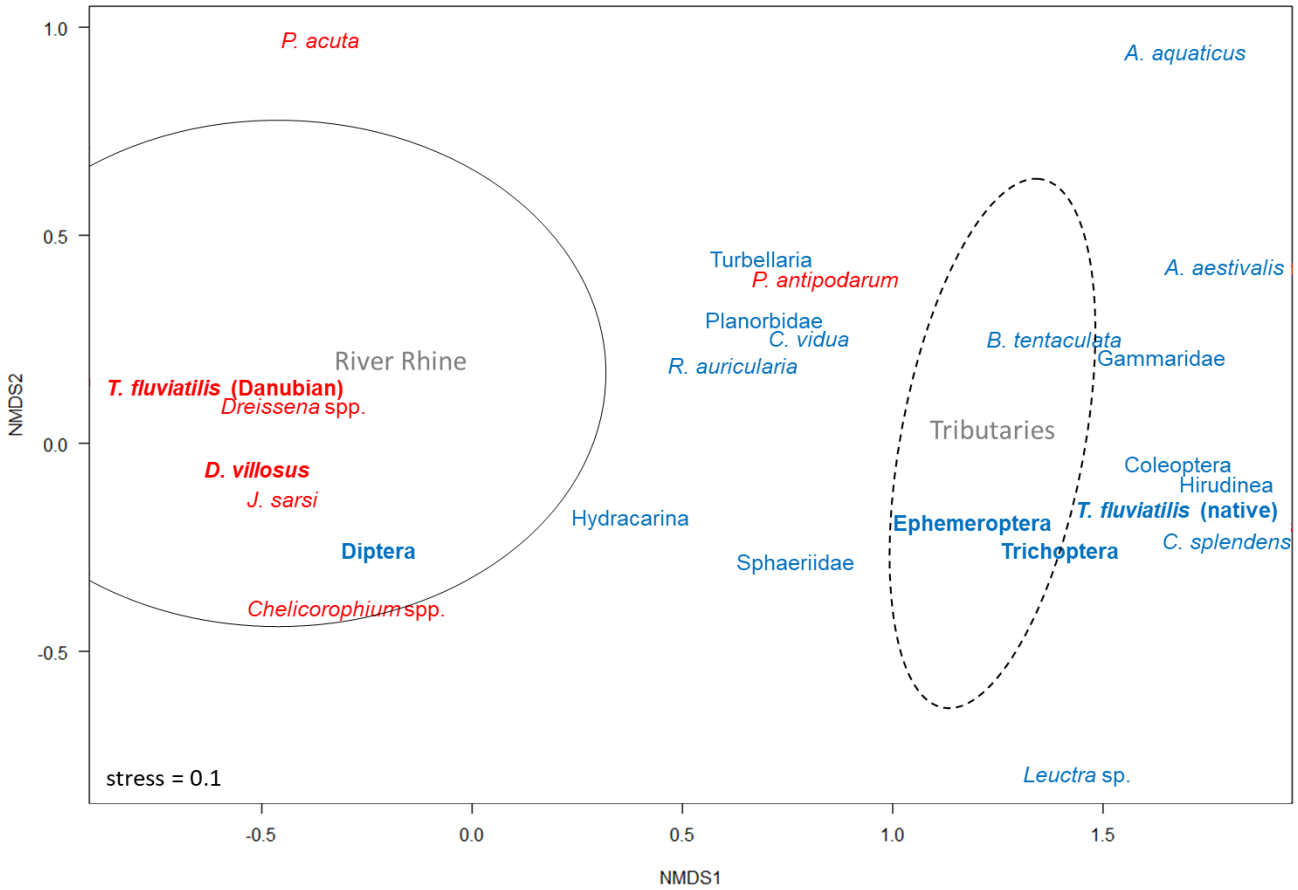


**Figure 3** Nonmetric Multidimensional Scaling (NMDS) biplot of the dissimilarity matrix (Bray-Curtis dissimilarity) of the densities of 25 macroinvertebrate taxa (18 native taxa marked in blue and seven non-native taxa marked in red) at two different types of sampling sites: at the River Rhine (n = 23) and at tributaries of the River Rhine system (Jagst, Kocher, and Tauber; n = 7). The taxa densities were added using weighted averages. Site scores are not shown for clarity. PERMANOVA analysis of the difference between the sites at the River Rhine and the tributaries showed a statistically significant difference (p = 0.001), and the taxa which mostly contribute to the overall dissimilarity (*simper()* analysis) are marked in bold.

## Discussion

The results of our analyses show that the geogenic originated factors calcium, sulphate, and strontium are the environmental parameters mostly correlating to the variability in the macroinvertebrate community. This is in contrast to our hypothesis assuming that metal pollution is a driving factor structuring the macroinvertebrate community, at least with regard to the sampling sites and taxa examined in our study. From the geogenic originated factors found to be relevant for the macroinvertebrate community structure, calcium is one of the most important mineral elements needed by all animals to maintain ionic balance and as integral parts of amino acids, nucleic acids, and structural compounds (Walker et al. 2012). Natural sulphate in surface waters originates from geogenic sources, i. e. water flow through rocks, or biogenic sources, e. g. the decomposition of organic matter (Jezierski et al. 2006). The metal strontium is a naturally occurring geogenic trace element found e. g. in the minerals celestite, strontianite, or strontium-rich aragonite, the latter being able to re-crystallise to pure calcite (Voutchkova et al. 2015). Hence, these factors, representative for the geogenic conditions at the respective sites, seem to have a stronger relevance for the analysed taxa than metal concentrations and the

other measured anthropogenic influenced contaminants, at least at the sampling sites in our study.

Among the anthropogenic influenced pollutants, we found significant correlations of the nitrogen compound nitrite to single taxa (Turbellaria and Sphaeriidae), but no significant relationships to the macroinvertebrate community in general. Nitrite can originate in freshwaters from anthropogenic sources like the agricultural use of fertilisers or wastewater discharge (Fent 2013). Regarding the analysed metals, only the concentrations of chromium were significantly correlated to the overall macroinvertebrate dataset. Chromium compounds originate from steel welding, chrome plating or the use of coatings, and are comparatively highly toxic as compared to other essential metals like copper or zinc (Campbell et al. 2009, Dhiman 2020). Although the measured concentrations of the metal chromium were significantly correlated to the overall macroinvertebrate taxa dataset, only the order Diptera was found to be significantly negatively correlated to its occurrence. For example, Hoekstra et al. (1994) compared the acute toxicity data of 196 different species for 26 chemicals, and emphasised the high sensitivity of the gastropod *P. acuta*, a taxon which occurs at our sampling sites as well, to chromium. Therefore, given the toxicity of chromium, the relevance of anthropogenically driven metal pollution for freshwater ecosystems in general, and the fact that our measurements of chromium, zinc, and copper concentrations in the River Rhine were at some of our sampling sites many times higher than the measurements of the actual River Rhine water quality report (IKSR/CIPR/ICBR 2021b), we would expect stronger significant effects of especially chromium and metal pollution in general on the identified taxa. However, it is also possible that not only inorganic pollutants like metals, but also e. g. organic toxicants that were not measured by us might be a relevant stressor for the macroinvertebrate community at our sampling sites (see also Schäfer et al. 2016).

In addition to the strong relationships between the geogenic originated factors and the macroinvertebrate community, our results show a significant separation between a community characterised by non-native taxa at the sampling sites in the River Rhine and a community characterised by native taxa in the tributaries Jagst, Kocher, and Tauber. Regarding the identified non-native macroinvertebrates, six out of seven taxa found at our sampling sites occurred exclusively in the River Rhine. Two non-native species, i. e. the invasive amphipod *D. villosus* and the Danubian form of the freshwater snail *T. fluviatilis*, showed the strongest contribution to the dissimilarity between the River Rhine community and that of the tributaries. In anthropogenically influenced rivers, the structural and physicochemical habitat alterations and environmental constraints favour euryoecious species with high competitive fitness, which leads to an increased colonisation by aquatic invasive species (Bij de Vaate et al. 2002, Früh et al. 2012, Leuven et al. 2009). Invaders are often more tolerant to environmental stressors like higher salt content, temperature, or organic pollution than native species (Karatayev et al. 2009, Leuven et al. 2009), and their invasion success can be attributed i. a. to biological characteristics like a less-specific food preference or the protection of juveniles (Bij de Vaate et al. 2002). Hence, if the invaders' new habitat is anthropogenically modified and stressed by pollution, as in the case of the River Rhine, tolerant invaders may either outcompete indigenous competitors or profit from unused resources after indigenous species have been exterminated from the habitat by various stressors (Bij de Vaate et al. 2002, Früh et al. 2012, Karatayev et al. 2009).

Simultaneously, though native species are often weakened by the ecological impairments and modifications in anthropogenically influenced

large rivers (Bij de Vaate et al. 2002), it is possible that they persist, e. g. along physiochemical gradients of heterogeneous habitats, or in tributaries (Gergs et al. 2013, Dick 2008, Palmer & Ricciardi 2004). The results of our study show a distribution focus of native taxa in the Jagst, Kocher, and Tauber, together with an absence of the identified non-native taxa. For the invasive amphipod *D. villosus*, which was the taxon that contributed the most to the separation of the macroinvertebrate communities, it is already known that it does not invade tributaries (Chen et al. 2012). In general, a lowered ability of many invasive taxa to colonise upstream sections and tributaries is assumed, but to our knowledge potential reasons are not known so far. However, the evident distribution focus of the non-native taxa in the River Rhine along with their effects on native taxa, resulting in a separation of species between the main river and the tributaries, seems to be a highly relevant factor structuring the macroinvertebrate community, at least at the sampling sites in our study. It has already been observed that community-level responses of stressors are well predicted by the dominance model, which assumes that the strongest stressor overrides the effects of other stressors (Morris et al. 2022). Hence, the occurrence of invasive species is a stressor potentially masking the effects of other stressors with smaller impacts, e. g. metal pollution, in our study.

In conclusion, our study indicates that the occurrence of non-native species could be a factor structuring the macroinvertebrate community to such a high extent that the effects of other stressors like metal contamination are potentially masked, which to our knowledge has not been shown to that degree so far. Nevertheless, we cannot fully exclude the importance of other stressors like organic pollution in our study, as their potential negative effects on macroinvertebrates are well known. Hence, for the management of freshwater systems and the achievement of their good ecological status, it is mandatory to consider different levels of stressors (i. e. abiotic as well as biological pressures and their interactions) in order to identify the most important management issues (Lemm et al. 2020, Ormerod et al. 2010).

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Supplementary material to

# Is metal pollution a relevant environmental factor affecting the macroinvertebrate community in the River Rhine system?

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**Table S1** Sampling sites and dates of data collection at the German Upper Rhine and the rivers Jagst, Kocher and Tauber, and coordinates.

| Site | River | Site name, specification | Coordinates (WGS84) | Sampling date (month/year) |
|---|---|---|---|---|
| S1 | | Grauelsbaum | 48°44'10"N, 7°58'08"E | 09/2019 |
| S2 | | Grauelsbaum, harbour | 48°44'38"N, 7°58'14"E | 09/2019 |
| S3 | | Greffern, ferry pier | 48°45'15"N, 7°58'15"E | 09/2019 |
| S4 | | Rheinmünster | 48°45'29"N, 7°59'41"E | 09/2019 |
| S5 | | Rheinmünster, marina | 48°45'23"N, 8°00'01"E | 09/2019 |
| S6 | | Rheinmünster, harbour | 48°45'35"N, 8°00'56"E | 09/2019 |
| S7 | | Söllingen | 48°47'18"N, 8°03'09"E | 09/2019 |
| S8 | | Iffezheim, lock | 48°50'03"N, 8°06'52"E | 09/2019 |
| S9 | Rhine | Plittersdorf, ferry pier | 48°53'16"N, 8°08'14"E | 09/2019 |
| S10 | | Steinmauern | 48°55'10"N, 8°09'56"E | 09/2019 |
| S11 | | Illingen | 48°56'24"N, 8°11'08"E | 09/2019 |
| S12 | | Au am Rhein | 48°57'26"N, 8°11'59"E | 09/2019 |
| S13 | | Neuburgweier, ferry pier | 48°58'37"N, 8°15'22"E | 08/2019 |
| S14 | | Neuburg | 48°59'12"N, 8°16'10"E | 09/2018 |
| S15 | | Daxlanden | 49°00'21"N, 8°17'41"E | 08/2019 |
| S16 | | Karlsruhe, harbour basin 1 | 49°00'59"N, 8°18'12"E | 08/2019 |
| S17 | | Karlsruhe, harbour basin 4 | 49°00'40"N, 8°19'57"E | 08/2019 |

| | | | | |
|---|---|---|---|---|
| S18 | | Karlsruhe | 49°02'07"N, 8°18'16"E | 09/2018 |
| S19 | | Eggenstein | 49°05'24"N, 8°21'13"E | 09/2018 |
| S20 | | Leimersheim | 49°07'17"N, 8°21'50"E | 08/2018 |
| S21 | | Speyer | 49°19'10"N, 8°26'59"E | 08/2018 |
| S22 | | Altrip | 49°26'03"N, 8°30'25"E | 08/2018 |
| S23 | | Mannheim | 49°32'37"N, 8°25'26"E | 08/2018 |
| S24 | Jagst | Herbolzheim | 49°16'59"N, 9°15'31"E | 09/2018 |
| S25 | | Ingelfingen | 49°17'53"N, 9°39'09"E | 09/2018 |
| S26 | Kocher | Ernsbach | 49°17'26"N, 9°30'53"E | 09/2018 |
| S27 | | Kochendorf | 49°13'39"N, 9°13'03"E | 09/2018 |
| S28 | | Igersheim | 49°29'30"N, 9°48'46"E | 09/2018 |
| S29 | Tauber | Lauda | 49°34'14"N, 9°42'28"E | 09/2018 |
| S30 | | Bronnbach | 49°41'56"N, 9°32'54"E | 09/2018 |

**Table S2** Site-specific measurement values of the 25 environmental parameters and the densities of 25 macroinvertebrate taxa at 30 sampling sites at the German Upper Rhine and the rivers Jagst, Kocher and Tauber. Minimum and maximum values of the respective variables are marked in bold. For units of the environmental variables see Table 1. Taxa densities are given in ind $m^{-2}$. For site descriptions see Table S1.

| Site | Temperature | Oxygen | pH | Conductivity | Chloride | Nitrite | Nitrate | Phosphate | Sulphate | Ammonium | Silicium | Calcium | Magnesium | Sodium | Biofilm_mass | Cu_water | Cr_water | Sr_water | Zn_water | Fe_water | Cu_biofilm | Cr_biofilm | Sr_biofilm | Zn_biofilm | Fe_biofilm |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 18.7 | 9.5 | 8.1 | 340 | 15.54 | 0.01 | 1.05 | 0.003 | 25.4 | 0.024 | 0.83 | 28.0 | 7.13 | 11.2 | 1.11 | **9.45** | **0.75** | 346 | 5.96 | **33.9** | 15.23 | 16.90 | 331 | 81.0 | 11265 |
| S2 | 18.2 | 8.1 | 8.2 | 340 | 15.91 | 0.010 | 1.03 | 0.005 | 25.1 | 0.027 | 1.00 | 30.6 | 7.10 | 11.6 | 0.82 | 5.58 | **0.75** | 346 | 6.01 | 37.2 | 21.52 | 21.04 | 342 | 93.2 | 13062 |
| S3 | 18.4 | 9.6 | 8.3 | 330 | 15.90 | 0.012 | 1.01 | 0.004 | 25.2 | 0.019 | 0.86 | 30.7 | 7.08 | 11.5 | 0.56 | 2.17 | **0.75** | 343 | 3.86 | 39.2 | 11.07 | 14.89 | 383 | 62.9 | 9930 |
| S4 | 20.9 | 8.9 | **7.7** | 340 | 13.76 | 0.013 | 0.92 | 0.004 | 24.5 | 0.023 | 0.77 | 29.8 | 7.06 | 10.1 | **3.69** | 5.38 | **0.75** | 347 | 6.80 | 54.4 | 16.83 | 19.47 | 318 | 136.0 | 13678 |
| S5 | 20.5 | 9.8 | **7.7** | 350 | 13.37 | 0.013 | 0.88 | 0.002 | 23.6 | 0.012 | 0.81 | 29.9 | 7.05 | **5.0** | 0.94 | 5.08 | **0.75** | 340 | 3.52 | 74.1 | 7.62 | 13.26 | 374 | 42.1 | 6547 |
| S6 | 20.5 | 9.1 | 8.0 | 350 | 14.06 | 0.012 | 0.91 | 0.003 | 24.0 | 0.021 | 0.90 | 28.5 | 7.08 | 10.1 | 1.54 | **1.20** | **0.75** | 346 | 3.67 | 42.5 | 14.21 | 18.35 | 451 | 81.0 | 11501 |
| S7 | 23.0 | **10.4** | 7.8 | 300 | 13.53 | 0.012 | 0.87 | 0.004 | 24.0 | 0.018 | 0.79 | 29.2 | 7.01 | **5.0** | 0.86 | **1.20** | **0.75** | 340 | 2.69 | 45.2 | 5.77 | **7.35** | 570 | 33.4 | 4990 |
| S8 | 20.3 | 8.6 | 7.8 | 330 | 13.57 | 0.007 | 0.93 | 0.007 | 24.0 | **0.030** | 1.12 | 31.4 | 7.13 | **5.0** | 1.72 | **1.20** | **0.75** | 344 | 11.81 | 86.5 | 24.48 | 21.24 | 345 | 95.2 | 13142 |
| S9 | 20.4 | 8.7 | 7.9 | 330 | 13.70 | 0.007 | 0.94 | 0.005 | 23.7 | 0.025 | 0.92 | 31.7 | 7.12 | **5.0** | 1.87 | **1.20** | **0.75** | 344 | 3.47 | 78.6 | 20.74 | 19.31 | 249 | 92.9 | 14127 |
| S10 | 20.9 | 9.0 | 7.8 | 300 | 13.68 | 0.010 | 0.97 | 0.006 | 22.6 | 0.024 | 1.05 | 30.4 | 6.99 | **5.0** | 1.70 | **1.20** | **0.75** | 333 | 3.86 | 82.4 | 21.23 | 19.66 | 259 | 96.7 | **14259** |
| S11 | 20.4 | 9.2 | 7.8 | 390 | **12.20** | 0.011 | 0.88 | 0.006 | **20.8** | 0.022 | 0.96 | **24.7** | **6.24** | **5.0** | 1.94 | **1.20** | **0.75** | 337 | 2.55 | 78.5 | 20.26 | 18.14 | 309 | 79.4 | 12643 |
| S12 | 19.9 | 8.7 | 7.8 | 330 | 13.76 | 0.011 | 0.89 | 0.004 | 22.4 | 0.020 | 1.16 | 28.2 | 7.0 | **5.0** | 1.76 | **1.20** | **0.75** | 331 | 2.20 | 53.3 | 18.19 | 11.36 | 349 | 49.1 | 6922 |
| S13 | 24.5 | 8.5 | 8.0 | 310 | 12.70 | 0.011 | 0.86 | 0.003 | 24.4 | 0.018 | 0.92 | 27.2 | 6.54 | **5.0** | 1.12 | **1.20** | **0.75** | 347 | 2.51 | 74.8 | 9.29 | 17.36 | 293 | 58.4 | 9042 |
| S14 | 21.0 | **6.0** | 8.0 | **230** | 18.05 | 0.011 | 1.04 | 0.017 | 25.9 | **0.006** | 1.35 | 51.7 | 6.85 | 13.5 | 0.93 | 1.35 | 2.95 | 298 | 5.16 | 329.0 | 9.49 | 18.29 | 197 | 61.6 | 10004 |
| S15 | 23.6 | 7.8 | 7.9 | 340 | 14.04 | 0.012 | 0.84 | 0.008 | 24.7 | 0.024 | 1.14 | 30.0 | 6.65 | **5.0** | 1.53 | **1.20** | **0.75** | 346 | 2.35 | 60.7 | 14.40 | 17.74 | 279 | 83.4 | 11066 |
| S16 | 24.2 | 8.4 | 8.4 | 330 | 13.24 | 0.010 | 0.62 | 0.002 | 25.0 | 0.015 | 0.36 | 28.6 | 6.79 | **5.0** | 1.19 | 2.40 | **0.75** | 345 | 6.04 | 89.0 | 13.39 | 17.43 | 176 | 69.4 | 9629 |
| S17 | 25.3 | 8.4 | 8.3 | 330 | 13.76 | 0.008 | **0.39** | 0.005 | 25.7 | 0.018 | **0.26** | 31.3 | 7.04 | **5.0** | 2.67 | **1.20** | **0.75** | 347 | **2.12** | 62.5 | 19.72 | **40.79** | 472 | **155.4** | 6283 |
| S18 | 24.3 | 6.8 | 8.0 | 279 | 18.49 | 0.010 | 1.08 | 0.010 | 27.2 | **0.006** | 1.15 | 54.2 | 6.93 | 14.1 | 1.99 | 1.35 | 2.56 | 318 | 3.04 | 173.9 | 9.56 | 16.61 | 293 | 47.9 | 8377 |

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S19 | 22.3 | 6.4 | 8.1 | 273 | 19.86 | 0.010 | 1.05 | 0.010 | 28.6 | **0.006** | 1.12 | 54.8 | 6.96 | 15.4 | 0.85 | 1.35 | 2.97 | 318 | 2.99 | 117.6 | 11.06 | 22.02 | 194 | 58.0 | 9197 |
| S20 | 25.2 | 7.2 | **9.0** | 288 | 18.69 | 0.007 | 0.77 | **0.001** | 26.7 | 0.018 | 1.32 | 45.1 | 7.36 | 14.7 | 0.53 | 1.35 | 2.85 | 321 | 3.33 | 176.8 | 10.23 | 14.72 | 196 | 39.6 | 6832 |
| S21 | 21.0 | 6.9 | 8.1 | 251 | 23.19 | 0.007 | 1.49 | 0.018 | 28.0 | 0.008 | 2.77 | 35.4 | 6.43 | 21.0 | 0.32 | 4.54 | **3.60** | **213** | **17.58** | **454.5** | **28.59** | 29.63 | **96** | 154.6 | 10394 |
| S22 | 25.4 | 7.0 | 8.0 | 283 | 19.16 | 0.005 | 0.77 | **0.001** | 27.3 | 0.008 | 1.45 | 45.5 | 7.40 | 15.1 | **0.18** | 1.35 | 2.86 | 324 | 3.13 | 165.1 | 6.07 | 11.83 | 415 | **30.7** | **4823** |
| S23 | **26.1** | 7.4 | 8.2 | 531 | 39.00 | 0.010 | 1.55 | 0.024 | 83.6 | 0.012 | 2.06 | 71.7 | 16.3 | 29.8 | 0.60 | 1.35 | 3.17 | 476 | 7.76 | 196.1 | 9.22 | 12.09 | 394 | 41.4 | 5398 |
| S24 | 16.3 | 8.9 | 8.7 | 710 | 50.17 | **0.002** | 3.69 | 0.022 | 196.7 | **0.006** | 2.37 | 191.7 | **20.0** | 29.8 | 0.27 | 1.35 | 1.66 | 1123 | 3.30 | 82.7 | 14.16 | 22.31 | 220 | 55.5 | 13817 |
| S25 | 18.7 | 7.2 | 8.9 | 849 | **66.02** | **0.030** | 2.29 | 0.067 | **200.0** | 0.019 | 2.46 | 197.0 | **20.0** | 44.5 | 1.24 | 1.35 | 2.20 | 1682 | 7.82 | 351.3 | 9.00 | 13.43 | 382 | 49.3 | 7356 |
| S26 | 18.7 | 8.7 | 8.9 | 855 | 63.36 | 0.007 | 1.74 | 0.067 | **200.0** | 0.007 | 1.90 | **200.0** | **20.0** | 44.9 | 0.32 | 1.35 | 1.23 | 1874 | 3.33 | 125.9 | **5.42** | 8.19 | **969** | 31.8 | 4956 |
| S27 | 18.3 | 9.0 | 8.6 | **856** | 63.40 | 0.005 | 1.72 | **0.075** | **200.0** | **0.006** | 2.24 | **200.0** | **20.0** | **46.2** | 0.31 | 1.35 | 1.22 | 1953 | 4.64 | 121.4 | 14.46 | 19.27 | 499 | 83.1 | 11496 |
| S28 | **15.4** | 7.3 | 8.0 | 727 | 52.14 | 0.027 | **6.33** | 0.060 | **200.0** | 0.011 | **3.78** | **200.0** | **20.0** | 26.0 | 0.62 | 1.35 | 1.87 | 1571 | 11.35 | 336.3 | 16.78 | 23.63 | 158 | 72.0 | 13146 |
| S29 | **15.4** | 8.6 | 8.1 | 772 | 43.17 | 0.009 | 5.84 | 0.043 | **200.0** | **0.006** | 3.46 | **200.0** | **20.0** | 24.6 | 1.46 | 1.35 | 1.34 | 1593 | 11.09 | 175.7 | 18.41 | 17.71 | 346 | 60.7 | 10444 |
| S30 | 15.7 | 8.7 | 8.2 | 829 | 34.33 | 0.011 | 5.91 | 0.040 | **200.0** | **0.006** | 3.08 | **200.0** | **20.0** | 18.5 | 0.94 | 1.35 | 1.40 | **2000** | 8.52 | 106.2 | 12.91 | 17.47 | 398 | 58.1 | 10175 |

| Site | Turbellaria | Hirudinea | Sphaeriidae | Planorbidae | *Bithynia tentaculata* | *Radix auricularia* | *Theodoxus fluviatilis* (native) | *Cypridopsis vidua* | *Asellus aquaticus* | Gammaridae | Hydracarina | Ephemeroptera | *Leuctra* sp. | Trichoptera | *Calopteryx splendens* | *Aphelocheirus aestivalis* | Coleoptera | Diptera | *Theodoxus fluviatilis* (Danubian) | *Potamopyrgus antipodarum* | *Physella acuta* | *Dreissena* spp. | *Jaera sarsi* | *Dikerogammarus villosus* | *Chelicorophium* spp. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 2 | **0** | **0** | **0** | **0** | **0** | **0** | 283 | 84 | **0** | **0** | 10 | 24 | 112 | **0** |
| S2 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 3 | **0** | **0** | **0** | 147 | 25 | **0** | **0** | 5 | 12 | 80 | 13 |
| S3 | **0** | **0** | 1 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 4 | 1 | **0** | 3 | **0** | **0** | **0** | 389 | 8 | **0** | **0** | 5 | 39 | 187 | 74 |
| S4 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 2 | 2 | **0** | **0** | **0** | **0** | **0** | 129 | 23 | **0** | **0** | **0** | 18 | 66 | **0** |
| S5 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 152 | 152 | **0** | **0** | 7 | **0** | 69 | **0** |
| S6 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 2 | **0** | **0** | 1 | **0** | **0** | **0** | 251 | 69 | **0** | **0** | 5 | 53 | 425 | **112** |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S7 | **0** | **0** | **0** | 2 | **0** | **0** | **0** | **0** | **0** | **0** | 1 | **0** | **0** | 13 | **0** | **0** | **0** | 337 | 31 | **0** | **0** | 13 | 35 | 347 | 24 |
| S8 | **0** | **0** | 2 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 6 | **0** | **0** | **0** | **0** | **0** | **0** | 445 | 17 | **0** | **0** | 4 | 13 | 69 | 43 |
| S9 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 3 | **0** | **0** | 78 | **0** | **0** | **0** | 309 | 65 | **0** | **0** | 3 | 107 | 453 | 1 |
| S10 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 5 | **0** | **0** | 47 | **0** | **0** | **0** | 291 | 41 | **0** | **0** | 16 | 87 | 181 | 5 |
| S11 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 43 | **0** | **0** | **0** | 75 | **546** | **0** | **0** | **1017** | **221** | 266 | 23 |
| S12 | **0** | **0** | **0** | 5 | **0** | **0** | **0** | **0** | **0** | **0** | 1 | **0** | **0** | 25 | **0** | **0** | **0** | 114 | 118 | 27 | 1 | 12 | 106 | **980** | 26 |
| S13 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 2 | **0** | **0** | 15 | **0** | **0** | **0** | 739 | 163 | **0** | **0** | 3 | 71 | 326 | 2 |
| S14 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 33 | **0** | **0** | **0** | **0** | 3 | **0** |
| S15 | **0** | **0** | 3 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 1 | **0** | **0** | 6 | **0** | **0** | **0** | 507 | 13 | **0** | **0** | 10 | 64 | 323 | 2 |
| S16 | **0** | **0** | 1 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 2 | 2 | **0** | 2 | **0** | **0** | **0** | 268 | 13 | **0** | **0** | 36 | 7 | 112 | 19 |
| S17 | 2 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 17 | 1 | **0** | 6 | **0** | **0** | **0** | 58 | 51 | 2 | **3** | 210 | 2 | 42 | **0** |
| S18 | 2 | **0** | **0** | **0** | **0** | 2 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 128 | 181 | 8 | **0** | 10 | 8 | 60 | **0** |
| S19 | 3 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 3 | 96 | **0** | 1 | 1 | 1 | 21 | 1 |
| S20 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 289 | **0** | **0** | 12 | 2 | 55 | **0** |
| S21 | **0** | **0** | **0** | **0** | **0** | 2 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 27 | 138 | **0** | **0** | 12 | 12 | 285 | **0** |
| S22 | **0** | **0** | **0** | **0** | 3 | **0** | **0** | 2 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 7 | 207 | **0** | **0** | 2 | 33 | 216 | **0** |
| S23 | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | 16 | 152 | 7 | 1 | 6 | 69 | 660 | 1 |
| S24 | 9 | 23 | **0** | **14** | **692** | **0** | 175 | **5** | **5** | 37 | 92 | 42 | **0** | 42 | 5 | **5** | **323** | 83 | **0** | **702** | **0** | **0** | **0** | **0** | **0** |
| S25 | **138** | **0** | **20** | **0** | 20 | 10 | 31 | 3 | **0** | **38** | 33 | 5 | **0** | 82 | **0** | **0** | 28 | 544 | **0** | 3 | **0** | **0** | **0** | **0** | **0** |
| S26 | 2 | 5 | 14 | 2 | 4 | 4 | 7 | 2 | **0** | 9 | 23 | 48 | 5 | 45 | **0** | **0** | 37 | 61 | **0** | 5 | **0** | **0** | **0** | **0** | **0** |
| S27 | **0** | 4 | **0** | **0** | 18 | **32** | 36 | 4 | **0** | 11 | 4 | 182 | **0** | 32 | **0** | 4 | 36 | 64 | **0** | 64 | **0** | **0** | **0** | **0** | **0** |
| S28 | 2 | **0** | 3 | 2 | 9 | **0** | 9 | **0** | 3 | 10 | **0** | 6 | **0** | 5 | **0** | **0** | 2 | 28 | **0** | 11 | **0** | **0** | **0** | **0** | **0** |
| S29 | **0** | **31** | **0** | **0** | 46 | **0** | **538** | **0** | **0** | **38** | **100** | 38 | **0** | 154 | **8** | **0** | 69 | 392 | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| S30 | 10 | **0** | 14 | **0** | 21 | **0** | 48 | **0** | **0** | **0** | 48 | **200** | **348** | **321** | **0** | **0** | 90 | **1052** | **0** | 28 | **0** | **0** | **0** | **0** | **0** |

# Paper 2

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# The Danubian cryptic invader *Theodoxus fluviatilis* (Gastropoda: Neritidae) in the River Rhine: a potential indicator for metal pollution?

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## Abstract

Metal pollution poses a major threat to aquatic systems especially in anthropogenic influenced areas, in as much as metals are persistent in the environment. The freshwater snail *Theodoxus fluviatilis* has often been used as an indicator species for the ecological status in river monitoring. In the River Rhine, the native Northern-European form of *T. fluviatilis* is nowadays extinct, whilst the Danubian form is spreading along the river. The aim of our study was to investigate if the cryptic invader is affected by metal exposure present in the River Rhine and to discuss its potential as an indicator for metal pollution. Several environmental abiotic (14 water environmental variables plus five common metal concentrations in water and biofilm) and biotic parameters (biofilm mass) were measured across 23 sites along the River Rhine. Five population and six histopathological parameters were evaluated on snails collected at all 23 sites. Aqueous chromium concentration was positively correlated to the damage of male reproductive organs of *T. fluviatilis*, and higher ammonium concentration was correlated to a decrease in snail size and an increase in the proportion of juveniles. None of the analysed snail parameters was negatively correlated to concentrations of other metals measured, like copper and zinc. Therefore, based on the parameters evaluated, our results indicate that the Danubian form of *T. fluviatilis* is only restrictedly suitable as an indicator for metal pollution in the River Rhine system. Further field and laboratory investigations including other stressors are necessary to evaluate the indicator potential of the cryptic invader holistically.

**Keywords** Chromium · Histopathological alterations · Population parameters · Gonads · River pollution

## Introduction

Metal pollution is an important factor affecting the health and safety of aquatic environments (Van Ginneken et al. 2017). Metals like copper, zinc, chromium, and cadmium enter surface waters due to anthropogenic use in many applications, e.g. in mining and metal refining industries. Metal ions are present in dissolved form or adsorbed to suspended matter, able to accumulate in sediments and biofilms, and not biodegradable (Morin et al. 2008; Walker et al. 2012). Although essential for organisms in trace quantities, they can be toxic to aquatic organisms even at low concentrations (µg/L; Van Ginneken et al. 2017; Walker et al. 2012), with an enormous variability of effects across metals and taxa (reviewed by Rainbow 2002).

The catchment area of the River Rhine is massively influenced by anthropogenic activities and pollution, due to its dense population and heavy industrialisation (Cioc 2002; Leuven et al. 2009; Uehlinger et al. 2009). Consequently, inorganic and organic pollutants emitted into the river ecosystem are multiple (e.g., metals, agrochemicals, nutrients, pharmaceuticals, surface paintings), both within point

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sources like sewage treatment plants, effluents of cooling systems, and industrial wastewater, as well as diffuse sources like land use, agricultural and urban runoff, and atmospheric deposition (Uehlinger et al. 2009). Given its intensive anthropogenic exploitation along with the resulting environmental contamination, the River Rhine is one of the most extensively monitored rivers in the world, with water quality being permanently surveyed by bordering countries and analyses published in public reports (as by the International Commission for the Protection of the Rhine, IKSR/CIPR/ICBR). Compared to the 1970s, the ecological status of the river improved due to comprehensive rehabilitation efforts including the improvement of water quality, restoration of riverine ecosystems and habitat connectivity (Leuven et al. 2009). Though, the current water quality of the River Rhine still does not meet the surface water quality standards as set by the Water Framework Directive, and metals still play an important role in river pollution, with differences at single locations (EU 2000; IKSR/CIPR/ICBR 2018; Sjerps et al. 2017). Hence, metal pollution in the River Rhine is still of importance on the spatial scale, also including areas beside the main stream of the river like industrial areas and harbours which are supposed to be a source for anthropogenic metal pollution (Daehne et al. 2017). However, even if effects of metals towards biota can be assumed at low concentration levels (Rainbow 2002; Walker et al. 2012), to our knowledge a multivariate field study investigating the potential importance of metals is missing, at least for the situation of the River Rhine.

Freshwater snails have often been reported as suitable indicators of metal pollution, since they are abundant in many types of freshwaters, have limited mobility and can accumulate large quantities of metals in their tissues (Das and Khangarot 2011; Dhiman and Pant 2021; Mahmoud and Abu Taleb 2013; Otludil and Ayaz 2020). The freshwater snail *Theodoxus fluviatilis* (Linné, 1758) is native to all large rivers in Central Europe (Bunje 2005; Zettler 2008) and prefers habitats with high oxygen and calcium contents and low salinity (Kangas and Skoog 1978; Zettler et al. 2004). Native populations of the Northern-European form of *T. fluviatilis* disappeared from the River Rhine in the late 1990s for unknown reasons (Westermann et al. 2007), and since 2006, specimens of *T. fluviatilis* phylogenetically originating from populations in the Danube and Black Sea drainages are found in the river (Bunje 2005; Gergs et al. 2015). The Danubian form of *T. fluviatilis* was denoted as cryptic invader (originally defined by Novak 2011), because it has presumably been introduced to the River Rhine by shipping through the Main-Danube canal (Gergs et al. 2015), as shown for several non-indigenous species originating from the Ponto-Caspian region (Alt et al. 2019; Bij de Vaate et al. 2002). It is observed to spread along the River Rhine since its discovery and to establish high population densities even in anthropogenically degraded habitats like industrial harbours (IKSR/CIPR/ICBR 2012; Rothmeier and Martens 2019). Whereas the Northern-European form of the snail has often been used as an indicator organism for the ecological status of the River Rhine, the suitability of the Danubian cryptic invader as indicator for environmental pollution in the River Rhine and possible effects on this particular form on the population and physiological level have not yet been evaluated.

The aim of our study is to examine if the Danubian form of *T. fluviatilis* is affected by metal exposure in the Upper River Rhine and to discuss its potential as indicator organism to detect adverse effects of metal pollution in the River Rhine. Resulting from its feeding behaviour almost exclusively on biofilms (Neumann 1961), *T. fluviatilis* is exposed to metals through the dietary pathway by grazing on contaminated biofilms and through aqueous exposure via gill respiration. Considering this and actual metal pollution in the River Rhine, negative effects on population and physiological parameters of the snail are hypothesised. We measured several abiotic (14 water environmental variables plus water and biofilm concentrations of the five common metals Cu, Cr, Sr, Zn, and Fe), and biotic parameters (biofilm mass) at 23 sites at the German Upper River Rhine. Five population and six histopathological parameters were examined on snails collected at all sites, as histopathology is an effective and suitable tool to analyse the effects of contaminants on aquatic organisms on the physiological level especially under field conditions (Otludil and Ayaz 2020).

## Methods and Materials

### Study sites and measured environmental parameters

We sampled 23 sites (S1-S23) located at the German Upper River Rhine, covering 116 km from river-km 316 to 432 (Fig. 1) from August to September 2018 and 2019, respectively. Sites were selected to presumably differ in their metal exposure conditions, being thus situated in different localities of the River Rhine (e.g., close to tributaries, ferry ports, or barrages, within industrial harbours and marinas). Sampling sites at the different localities were chosen to be comparable with respect to visual environmental characteristics (i.e., substrate, occurrence of aquatic macrophytes, water depth) to minimise their potential impact (for further site details see Table S1 in supplementary). At each site, 25 abiotic and biotic environmental parameters were measured (Table 1). Water temperature,

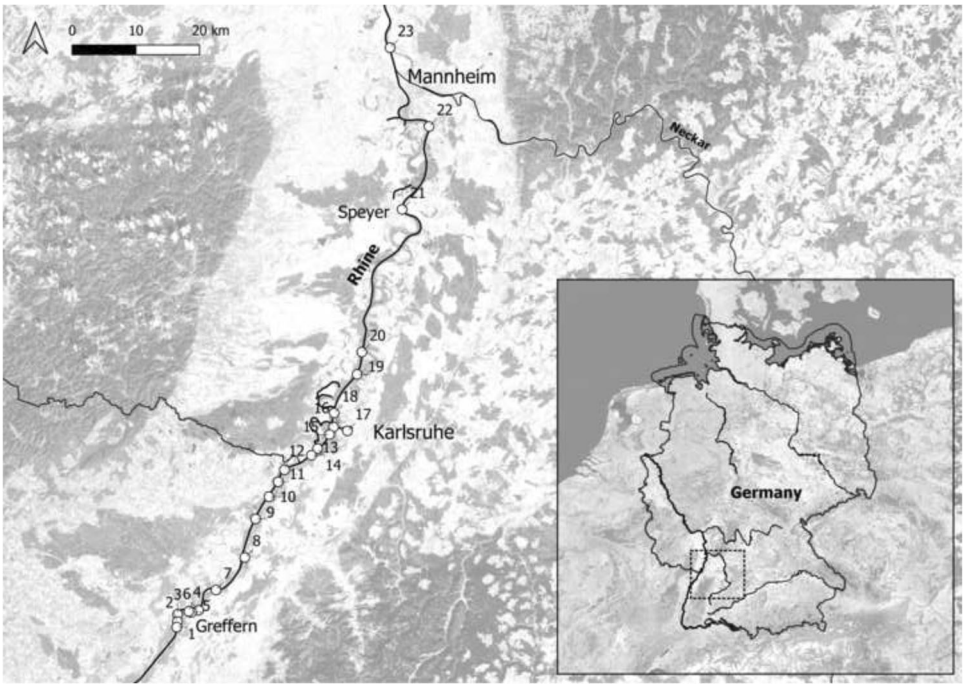


**Fig. 1** Map of study area showing Germany and its bigger 1st order rivers in overview (large-scale map) and the 23 sampling sites at the Upper River Rhine (detail map). Sampling locations are marked by dots with respective site numbers (for coordinates and further details see Table S1). Map created with the program QGIS (QGIS Development Team 2020)

dissolved oxygen, pH, and electrical conductivity (using DO-100, DO-100, PH-100 ATC and LWT-01, respectively, Voltcraft, Switzerland) were measured directly in the field. Water samples (1 L) were transported to the laboratory in polypropylene bottles. A subsample of 250 ml was frozen at −22 °C for nutrient measurements until further processing. Nutrients (chloride [$Cl^-$], nitrite [$NO_2^-$-N], nitrate [$NO_3^-$-N], phosphate [$PO_4^{3-}$-P], sulphate [$SO_4^{2-}$], ammonium [$NH_4^+$-N], calcium [$Ca^{2+}$], magnesium [$Mg^{2+}$], sodium [$Na^+$], and silicium [Si]) were analysed from 0.45 µm filtered water samples using continuous-flow-analysis (SAN++, Skalar, The Netherlands) and ion chromatography (Metrohm, Switzerland). Water subsamples for metal analyses (total volume 40 ml) were filtered (0.45 µm) and acidified with ultrapure nitric acid (Merck, Germany) to a final concentration of 0.5% until analysis. At each site, a sample of biofilm was scraped from a defined stone surface with a spatula, stored in river water (total volume 10 ml) and also acidified (ultrapure nitric acid, Merck, Germany, final concentration of 0.5%). Water and biofilm concentrations of five metals (copper [Cu], chromium [Cr], zinc [Zn], strontium [Sr], and iron [Fe]) were quantified using inductively coupled plasma optical emission spectrometry (Perkin Elmer, USA; for details on analytical methods see Rothmeier et al. 2020). To determine periphyton biomass, five subsamples per site were filtered on precombusted glass-fibre filters (Whatman GF6, Ø 25 mm, Maidstone, UK) for ash-free dry mass (AFDM) and dried at 105 °C for 24 h. After weighing (dry mass), the filters were combusted at 550 °C for 8 h and weighed again; the AFDM was calculated by subtraction.

## Sampling of *T. fluviatilis* and measured biological parameters

To assess *T. fluviatilis* biological variables, ten randomly chosen riprap stones were collected at each site, for sampling of snails attached to the surface (total n = 1.650 snails; range at sites 11–314 snails: large range due to extremely small (< 20) numbers at S3, S4, S8, S15, and S16, and an extremely large (> 300) number at S11). Five population (snails and egg density, shell size, and female and juvenile percentage) and six histopathological parameters (haemocyte infiltration, midgut gland, kidney and gill dilatation, and female and male gonads pathology) were analysed (Table 2) to determine the individual and population physiological condition of *T. fluviatilis* across sites. To estimate mean snail (individuals $m^{-2}$) and egg (eggs $m^{-2}$) density, the number of snails and egg capsules on their shells was first counted. Then, to estimate stones surface area, stones were wrapped in aluminium foil: by weighing reference aluminium foil pieces, the function of foil weight to corresponding surface value was calculated. Mean snail and egg density were calculated from the ten sampled stones per site (Table 2). Shell length of each individual was measured with a digital calliper (L826.1, Roth, Germany) to the nearest 0.1 mm to calculate mean shell size at sites from all sampled individuals (Table 2). Based on expert judgement (B. Waterman, personal communication), snails with a maximum shell length of 4 mm were considered as premature, in order to calculate the proportion of juvenile snails per site from all individuals (Table 2). A subsample of 13–46 adult snails per site (depending on sufficient

**Table 1** Environmental parameters measured at the 23 sampling sites of the German Upper River Rhine and their respective measured minimum-maximum ranges

| Compartment | Environmental variable | Unit | Range |
|---|---|---|---|
| Water | Temperature | °C | 18.2–26.1 |
| | pH | – | 7.7–9.0 |
| | Conductivity | µS $cm^{-1}$ | 230–531 |
| | Dissolved oxygen | mg $L^{-1}$ | 6.0 –10.4 |
| | Chloride ($Cl^-$) | mg $L^{-1}$ | 12–39 |
| | Nitrite ($NO_2^-$-N) | mg $L^{-1}$ | 0.005–0.013 |
| | Nitrate ($NO_3^-$-N) | mg $L^{-1}$ | 0.4–1.6 |
| | Phosphate ($PO_4^{3-}$-P) | mg $L^{-1}$ | 0.001–0.024 |
| | Sulphate ($SO_4^{2-}$) | mg $L^{-1}$ | 21–84 |
| | Ammonium ($NH_4^+$-N) | mg $L^{-1}$ | 0.006–0.030 |
| | Calcium ($Ca^{2+}$) | mg $L^{-1}$ | 25–72 |
| | Magnesium ($Mg^{2+}$) | mg $L^{-1}$ | 6.2–16.3 |
| | Sodium ($Na^+$) | mg $L^{-1}$ | 5.0–29.8 |
| | Silicium (Si) | mg $L^-1$ | 0.3–2.8 |
| | Copper (Cu) | µg $L^{-1}$ | 1.2–9.5 |
| | Chromium (Cr) | µg $L^{-1}$ | 0.8–3.6 |
| | Strontium (Sr) | µg $L^{-1}$ | 213–476 |
| | Zinc (Zn) | µg $L^{-1}$ | 2.1–17.6 |
| | Iron (Fe) | µg $L^{-1}$ | 34–455 |
| Biofilm | Copper (Cu) | mg $kg^{-1}$ | 5.8–28.6 |
| | Chromium (Cr) | mg $kg^{-1}$ | 7.4–40.8 |
| | Strontium (Sr) | mg $kg^{-1}$ | 96–570 |
| | Zinc (Zn) | mg $kg^{-1}$ | 31–155 |
| | Iron (Fe) | mg $kg^{-1}$ | 4,823–14,259 |
| | Biomass of periphyton (AFDM) | mg $cm^{-2}$ | 0.2–3.7 |

For detailed measurement values see Supplementary Table S2

availability of snails with shell length > 4 mm) was transferred into bottles with river water from the respective site and transported to the laboratory for histopathological analysis (in total 543 specimens; methodology see Rothmeier et al. 2020). Examination included determination of sex to calculate the proportion of females per site and analysis of the proportion of snails per site affected by pathological findings in tissues and organs, which were haemocyte infiltrations, midgut gland, kidney, or gill dilatations, as well as male gonad pathology (i.e., dilatations in male gonads or accessory glands, and stopped spermatogenesis) and female gonad pathology (i.e., dilatations in female gonads or accessory glands, and stopped oogenesis) (Table 2; Rothmeier et al. 2020). These tissues and organs are commonly investigated in histopathology, because a magnitude of studies indicate pathological changes and/or deformations as responses to a variety of environmental stressors (e.g., Otludil and Ayaz 2020; Rothmeier et al. 2020; Watermann et al. 2008).

**Table 2** Population and histopathological parameters of the Danubian form of *Theodoxus fluviatilis* (total $n = 1.650$) collected at the 23 sampling sites of the German Upper River Rhine and their respective measured minimum-maximum ranges

| | Biological variable | Unit | Range |
|---|---|---|---|
| Population | Mean density | ind $m^{-2}$ | 8–546 |
| | Mean shell size | mm | 3.5–7.5 |
| | Mean density of eggs | eggs $m^{-2}$ | 0–558 |
| | Female individuals | % | 16.7–77.3 |
| | Juvenile individuals | % | 4.6–78.3 |
| Histopathology | Haemocyte infiltration | % | 0–25 |
| | Midgut gland dilatation | % | 0–50 |
| | Kidney dilatation | % | 0–6.5 |
| | Gill dilatation | % | 0–16.7 |
| | Males: gonads pathology | % | 0–25 |
| | Females: gonads pathology | % | 0–89 |

For detailed measurement values see Supplementary Table S2

## Data analysis

Water metal concentrations which were lower than the limit of quantification (LOQ) were calculated as LOQ/2 (Clarke 1998) and those which were lower than the limit of detection (LOD) were taken as zero values (LOQ/LOD: Cu 2.2/0.7 µg/L, Cr 0.7/0.2 µg/L, Sr 0.02/0.01 µg/L, Zn 1.1/0.3 µg/L, Fe 0.8/0.2 µg/L). Abiotic and biotic environmental (Table 1) and biological (population and histopathological) variables of snails (Table 2) were standardised to zero mean and unit variance for dimensionally heterogenous variables. To improve their distribution, the skewed and widespread environmental variables were log(x) transformed prior to analysis (Borcard et al. 2018). Biological *T. fluviatilis* variables were Hellinger-transformed to give low weights to variables with low counts and many zeros (Legendre and Gallagher 2001).

Distance-based redundancy analysis (db-RDA), a constrained ordination method which allows to calculate a dissimilarity matrix of every distance measure (Legendre and Anderson 1999), was used to examine the effects of the abiotic and biotic environmental variables on the five population and six histopathological parameters of *T. fluviatilis*. Since biological *T. fluviatilis* variables contained values of zero in egg density and histopathological variables, Bray-Curtis dissimilarity was used as distance measure. The environmental variables' variance inflation factors (VIFs) were computed, as strong linear dependencies (autocorrelations) are possible in a large set of explanatory variables. As all environmental variables showed VIF > 10, which indicates strong collinearity (Borcard et al. 2018), a

variable selection procedure was conducted: the function *bioenv()* of the R package vegan (version 2.5–7, Oksanen et al. 2020) was used to select the subset of scaled environmental variables whose Euclidean distances had the maximum (rank) correlation to the response dissimilarity matrix (as suggested by Clarke and Ainsworth 1993). Significance of the db-RDA results was analysed by permutation tests using the function *anova()* of vegan (999 permutations). Finally, the univariate approach of Generalised Linear Models (GLM, error distribution = Gaussian) was used to determine the significance of the relationships between single *T. fluviatilis* biological parameters and selected environmental variables. All calculations and statistical analyses were conducted with the program R (version 3.6.3, R Core Team 2020).

## Results

### General description of the environmental and biological measured parameters

At all of the 23 examined sites, concentrations of the five analysed metals (Cu, Cr, Sr, Zn, and Fe) were measured in filtered water and biofilm samples above the LOD. Highest aqueous metal concentrations were found at sites in the main stream, for copper near the city of Grauelsbaum (S1), for chromium, zinc, and iron near the city of Speyer (S21), and for strontium near the city of Mannheim (S23; Table S2). Measured metal concentrations in biofilm samples were highest in the industrial harbour of Karlsruhe for chromium and zinc (S17) and in the main stream near the cities of Söllingen for strontium (S7), Steinmauern for iron (S10), and Speyer for copper (S21; Table S2). The highest concentrations of chloride, nitrate, phosphate, sulphate, calcium, magnesium, and sodium were measured near the city of Mannheim (S23; Table S2). The lowest nitrite concentrations were found at the sites near the cities of Grauelsbaum and Rheinmünster (S1, S4, and S5), and the highest near the city of Altrip (S22; Table S2). At the sampling sites near the cities of Neuburg, Karlsruhe, and Eggenstein (S14, S18, and S19), the lowest ammonium concentrations were measured, whereas the highest ammonium concentration was measured near the lock at Iffezheim (S8; Table S2).

Specimens of the Danubian form of *T. fluviatilis* were present at all sampling sites, showing densities with up to 546 individuals (ind) $m^{-2}$, with densities ranging from 8 ind $m^{-2}$ at a ferry pier near the city of Greffern (S3) to 546 ind $m^{-2}$ in the main stream near the city of Illingen (S11; Table S2). At 22% (5 out of 23) of the examined sites, no egg capsules of *T. fluviatilis* were found on the snail's shells, whereby highest density of eggs was found in a pleasure boat marina near the city of Rheinmünster (S5, 588 eggs $m^{-2}$; Table S2). The proportion of juvenile snails was lowest in the aforementioned pleasure boat marina (4.6%, S5), with showing a wide range over sampling sites up to 78.3% near the city of Illingen (S11; Table S2). Histopathologic alterations in organs of *T. fluviatilis* were found at all of the examined sites. Most frequent findings were pathologic alterations of female reproductive organs (gonads and accessory glands) at 70% (16 out of 23) of sites and dilatations in midgut glands at 65% (15 out of 23) of sites. Less frequent were pathologic dilatations of snail's gills at 4% (1 out of 23) of sites and kidneys at 13% (3 out of 23) of sites.

### Correlation between snail and environmental parameters

The variable selection procedure showed that water concentrations of ammonium and chromium were the most influential environmental variables in correlation to *T. fluviatilis* biological parameters, based on the parameters evaluated. The db-RDA model of *T. fluviatilis* data constrained by these two explanatory variables showed statistical significance of the global canonical relationship without collinearity of variables ($p_{\text{global model}} < 0.01$, $R^2_{\text{adj}} = 10.2$; Fig. 2). All other biotic and abiotic environmental variables showed no significant correlation to the biological snail parameters.

In the parsimonious db-RDA model with the selected variables, aqueous ammonium concentration had a statistically significant relationship to biological *T. fluviatilis* variables ($p = 0.01$; Table 3a). The proportion of juvenile snails was significantly higher and the mean snail shell size significantly smaller at higher ammonium concentrations (GLM, $p < 0.01$ and $< 0.05$, respectively; Table 3b). Pathologic alterations in gonads of male snails and impairment of spermatogenesis were significantly more often found at higher aqueous chromium concentrations (GLM, $p < 0.01$; Table 3b, Fig. 3).

## Discussion

Our hypothesis presuming negative effects of metal pollution on the Danubian form of *T. fluviatilis* in the German Upper River Rhine can only be partly confirmed. According to our results based on the parameters evaluated, the introduced cryptic invader is significantly affected by two factors: exposure towards aqueous chromium and ammonium concentrations. Thus, none of the analysed population and histopathological parameters of Danubian *T. fluviatilis* in our study was negatively affected by any of the measured metal concentrations in water and biofilm except chromium.

However, as metal speciation can be an important criterion to analyse the toxicity of metals for aquatic biota (Di Toro et al. 2001; Santore et al. 2001), and considering the fact that water parameters in our study were exclusively measured from filtered water samples, our results are limited to the specification of our study design. Hence, more investigations also regarding concentrations of metal ions bound to particles are necessary to evaluate effects of metal exposure holistically.

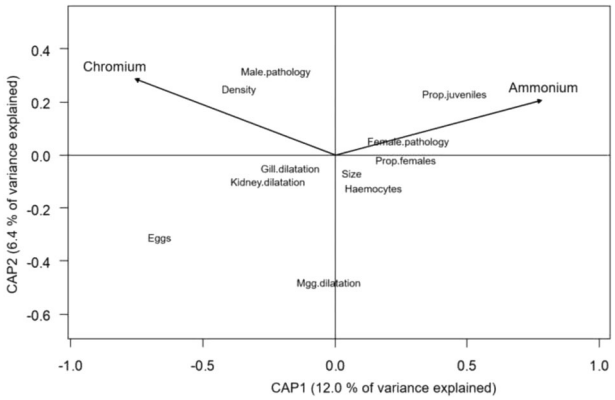


**Fig. 2** Distance-based redundancy analysis (db-RDA) correlation biplot of the first two canonical axes of *T. fluviatilis* (Danubian form) population and histopathological variables (see Table 2) after *bioenv()* selection of the constraining environmental variables ammonium and chromium concentrations in water (see Table 3). Site scores ($n = 23$) not displayed for clarity, scaling 2 biplot. Ordination based on Bray-Curtis dissimilarity and Hellinger-transformed *T. fluviatilis* biological variables. Abbreviations of constrained *T. fluviatilis* biological variables: Density = mean snail density (ind $m^{-2}$), Eggs = mean egg density (eggs $m^{-2}$), Female.pathology = dilatations in female gonads or accessory glands, and stopped oogenesis, Gill.dilatation = pathologic damage of gill tissue, Haemocytes = pathologic haemocyte infiltrations in tissues, Kidney.dilatation = pathologic damage of kidney tissue, Male.pathology = dilatations in male gonads or accessory glands, and stopped spermatogenesis, Mgg.dilatation = pathologic damage of midgut gland tissue, Prop.females = proportion of female snails, Prop. juveniles = proportion of juvenile snails, Size = mean shell size (mm)

At the examined sites in the Upper Rhine, which were partly close to industry locations, chromium exposure was connected to pathologic alterations in male reproductive organs of Danubian *T. fluviatilis*. Playing an important role in environmental pollution in general due to its wide use in many industries like steel welding, mining, or coating applications (Dhiman 2020; Sivakumar et al. 2014), chromium is one of the more toxic metals for biota. Its toxicity cannot be reduced by protein binding and accumulation within organisms to reduce its bioavailability, therefore it needs to be completely detoxified or excreted (Rainbow 2002; Sivakumar et al. 2014). Moreover, especially hexavalent compounds of chromium ($Cr^{6+}$) have in many laboratory studies shown to be highly toxic for animals, leading to negative effects of chromium exposure on male reproductive systems including morphological damage, altered testicular biochemistry or decreases in testis proteins (summarised by Campbell et al. 2009). Toxicity studies using the terrestrial snail *Helix aspersa* demonstrated acute chromium exposure to cause increased mortality and electrolyte disturbance in test animals (24h-$LC_{50}$ 15.13 mg/L, Dhiman 2020). The findings in our study indicate its toxicity to be of a relevance for aquatic snails as well. Given the fact that the effects of water chromium concentration on snails in our study were of a higher importance compared to the other measured metal concentrations, it is presumed to be a factor which has to be

**Table 3** **a** Results of permutation tests (999 permutations) of parsimonious distance-based RDA model after *bioenv()* selection of the two variables ammonium and chromium water concentration constraining five population and six histopathological variables of *T. fluviatilis* (Danubian form). Variance inflation factors (VIF) < 10 show no collinearity of variables. **b** Results of Generalised Linear Model (GLM) analysis showing relationships between ammonium and chromium water concentrations and single *T. fluviatilis* population and histopathological variables

| Selected environmental variables | Ammonium | | | Chromium | | |
|---|---|---|---|---|---|---|
| **a Distance-based RDA** | | | | | | |
| Anova (999 permutations) | F | *p*-value | VIF | *F* | *p*-value | VIF |
| | 2.85 | **0.01** | 2.94 | 1.66 | 0.09 | 2.94 |
| **b Generalised Linear Model** | | | | | | |
| *T. fluviatilis* biological variables | coeff | *p*-value | | coeff | *p*-value | |
| Mean density (ind $m^{-2}$) | −2904 | 0.43 | | 28 | 0.26 | |
| Mean shell size (mm) | −65 | **0.03** | | 0.13 | 0.52 | |
| Mean no. of eggs (eggs $m^{-2}$) | −6195 | 0.12 | | 18 | 0.51 | |
| Female individuals (%) | 526 | 0.23 | | −2.1 | 0.49 | |
| Juvenile individuals (%) | 1547 | **0.009** | | −4.6 | 0.28 | |
| Haemocyte infiltration (%) | 79 | 0.63 | | −1.2 | 0.26 | |
| Midgut gland dilatation (%) | −355 | 0.37 | | −1.5 | 0.58 | |
| Kidney dilatation (%) | −108 | 0.05 | | 0.52 | 0.16 | |
| Gill dilatation (%) | −165 | 0.11 | | 1.01 | 0.15 | |
| Males: gonads pathology (%) | −316 | 0.21 | | 4.9 | **0.002** | |
| Females: gonads pathology (%) | −59 | 0.94 | | −2.8 | 0.59 | |

Bold values indicate significant effects ($p < 0.05$).

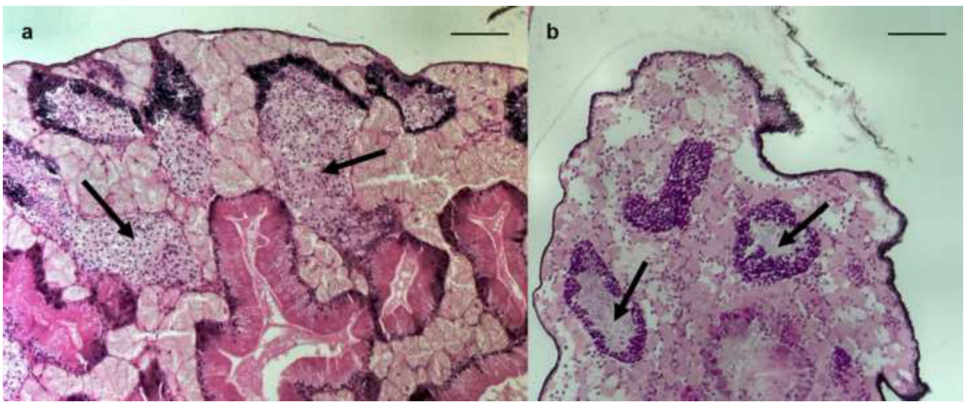


**Fig. 3** Microscope photographs of histologically prepared sections (2–3 µm) from tissues of the Danubian form of the freshwater snail *Theodoxus fluviatilis* from the River Rhine. Counter-staining with haematoxylin and eosin, bar 50 µm. Photographs showing male snail testis with **a** resorption of spermatogoniae (arrows), **b** arrested ( = stopped) spermatogenesis (arrows)

considered for acting upon organisms in the anthropogenically influenced environment of the River Rhine. Dissolved aqueous chromium concentrations measured at sampling sites in our study (0.8−3.6 µg/L, Table 1) are partly higher than concentrations of < 0.2−0.33 µg/L reported by the IKSR/CIPR/ICBR (2018). Although annual chromium concentrations in the River Rhine are significantly lower than the national environmental quality standard for surface waters (640 µg/L, IKSR/CIPR/ICBR 2018), the importance of a continuous surveillance of the metal in the river, keeping in mind the negative effects of chromium concentration on *T. fluviatilis* according to the results of our study, is emphasised.

The positive correlation between higher ammonium concentrations and smaller snail size and higher proportion of juvenile *T. fluviatilis* in this study can presumably be explained by a direct effect. Ammonia is a known degradation product of organic matter and therefore a potential chemical indicator for eutrophication in freshwater systems (Zaghloul et al. 2019), which in turn can lead to higher primary productivity. As *T. fluviatilis* is a nearly exclusive biofilm grazer feeding on diatoms (Neumann 1961), a higher food supply due to a higher amount of the current total biofilm biomass at sites with higher ammonium concentrations is likely to lead to increased reproduction and therefore a higher proportion of smaller and juvenile snails.

Due to their wide use in metal industries or as components of biocides and therefore relevant emission into surface waters, the metals copper and zinc play an important role for environmental pollution (Walker et al. 2012). In a number of European marinas, copper concentrations were too high for the approval of copper-based antifouling paints (Lagerström et al. 2020; Ytreberg et al. 2021). Although copper and zinc are essential trace metals being components of enzymes and proteins in organisms (Rainbow 2002), toxicity occurs when threshold metal concentrations are exceeded, but also in function of the organism, its body mass and metal bioavailability (Fent 2004; Van Ginneken et al. 2017). In general, water concentrations of copper and zinc in the River Rhine are below national environmental quality standards for surface waters (160 µg/L for copper and 800 µg/L for zinc). Though, measured dissolved aqueous copper and zinc concentrations at sampling sites in our study (1.2−9.5 µg/L Cu and 2.1−17.6 µg/L Zn; Table 1) were hypothesised to have a potential negative effect on biological parameters of Danubian *T. fluviatilis*, as they exceed values of comparable studies. They were partly higher than concentrations of the IKSR monitoring (0.77 −2.4 µg/L Cu and < 1−5.3 µg/L Zn; IKSR/CIPR/ICBR 2018), which could be due to the fact that sites with a potential higher metal exposure, like marinas or industrial harbours, were selected. Furthermore, both copper and zinc concentrations in our study were at individual sites higher than concentrations found in a comparable study in Swedish harbours leading to higher mortality, reduced growth, and lower fecundity of the brackish water form of *T. fluviatilis* (2.7−3.7 µg/L Cu and 7.1−10.6 µg/L Zn; Bighiu et al. 2017). Given our results showing no significant effects of detected copper and zinc concentrations on population and histopathological parameters of the Danubian form of *T. fluviatilis* in the field, it can be presumed that the cryptic invader is able to cope with concentrations of these metals at the examined sites. This assumption is supported by the fact that, considering copper concentrations, our measurements are lower than the 21-day $LC_{50}$ for copper of 16 µg/L derived from a laboratory study with the Danubian form of *T. fluviatilis* (Rothmeier et al. 2020). Furthermore, the newly introduced snail shows high population densities up to more than 500 individuals $m^{-2}$ (Table 2), with a density of more than 100 individuals $m^{-2}$ at site S21 where aqueous chromium, zinc, and iron concentrations were highest (Table S2). Due to this lack of sensitivity, we conclude that the Danubian form of *T. fluviatilis* is only restrictedly suitable as an indicator considering at least metal pollution in

light of the parameters evaluated in our field study in the River Rhine.

However, one must also consider the enormous variability of effects of not only metals, but also other environmental pollutants, across metals and invertebrate taxa. Snails have various detoxification mechanisms to counteract metal toxicity and reduce their bioavailability (Bighiu et al. 2017; Mahmoud and Abu Taleb 2013; Watermann et al. 2008), which is not the case for several other sensitive taxa. The knowledge of effects on a chosen species is essential for environmental monitoring, but concentrations should be compared considering the whole variety of invertebrate species, also taking the transfer of metals along food chains and the community level into account (Rainbow 2002). Furthermore, the difference in sensitivity between indigenous and invading species, as well as intraspecific variability, may play an important role in the future.

## Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Author contribution** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Louisa Marie Rothmeier, René Sahm, Burkard Watermann, and Jennifer Bartz. The first draft of the manuscript was written by Louisa Marie Rothmeier and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

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Supplementary material to

# The Danubian cryptic invader *Theodoxus fluviatilis* (Gastropoda: Neritidae) in the River Rhine: a potential indicator for metal pollution?

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**Table S1:** Sampling sites (number, km, name, and coordinate) and dates of sample collection at the German Upper River Rhine.

| No. | Km | Name | Coordinate (WGS84) | Sampling date (month/year) |
|---|---|---|---|---|
| 1 | 316 | Grauelsbaum | 48°44'10"N, 7°58'08"E | 09/2019 |
| 2 | 317 | Grauelsbaum, harbour | 48°44'38"N, 7°58'14"E | 09/2019 |
| 3 | 318 | Greffern, ferry pier | 48°45'15"N, 7°58'15"E | 09/2019 |
| 4 | 320 | Rheinmünster | 48°45'29"N, 7°59'41"E | 09/2019 |
| 5 | 321 | Rheinmünster, marina | 48°45'23"N, 8°00'01"E | 09/2019 |
| 6 | 322 | Rheinmünster, harbour | 48°45'35"N, 8°00'56"E | 09/2019 |
| 7 | 328 | Söllingen | 48°47'18"N, 8°03'09"E | 09/2019 |
| 8 | 334 | Iffezheim, lock | 48°50'03"N, 8°06'52"E | 09/2019 |
| 9 | 340 | Plittersdorf, ferry pier | 48°53'16"N, 8°08'14"E | 09/2019 |
| 10 | 345 | Steinmauern | 48°55'10"N, 8°09'56"E | 09/2019 |
| 11 | 347 | Illingen | 48°56'24"N, 8°11'08"E | 09/2019 |
| 12 | 349 | Au am Rhein | 48°57'26"N, 8°11'59"E | 09/2019 |
| 13 | 354 | Neuburgweier, ferry pier | 48°58'37"N, 8°15'22"E | 08/2019 |
| 14 | 355 | Neuburg | 48°59'12"N, 8°16'10"E | 09/2018 |
| 15 | 359 | Daxlanden | 49°00'21"N, 8°17'41"E | 08/2019 |
| 16 | 360 | Karlsruhe, harbour basin 1 | 49°00'59"N, 8°18'12"E | 08/2019 |
| 17 | 360 | Karlsruhe, harbour basin 4 | 49°00'40"N, 8°19'57"E | 08/2019 |
| 18 | 362 | Karlsruhe | 49°02'07"N, 8°18'16"E | 09/2018 |
| 19 | 369 | Eggenstein | 49°05'24"N, 8°21'13"E | 09/2018 |
| 20 | 372 | Leimersheim | 49°07'17"N, 8°21'50"E | 08/2018 |
| 21 | 400 | Speyer | 49°19'10"N, 8°26'59"E | 08/2018 |
| 22 | 414 | Altrip | 49°26'03"N, 8°30'25"E | 08/2018 |
| 23 | 432 | Mannheim | 49°32'37"N, 8°25'26"E | 08/2018 |

**Table S2:** Detailed measurement values of environmental parameters (Table 1) and *Theodoxus fluviatilis* biological variables (Table 2). Minimum and maximum values of respective variables are marked in bold.

| Site_No | Temperature | pH | Conductivity | Oxygen | Chloride | Nitrite | Nitrate | Phosphate | Sulphate | Ammonium | Silicium | Calcium | Magnesium | Sodium | Cu_water | Cr_water | Sr_water | Zn_water | Fe_water | Cu_biofilm | Cr_biofilm | Sr_biofilm | Zn_biofilm | Fe_biofilm | Biofilm_mass |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 18.7 | 8.1 | 340 | 9.5 | 15.54 | **0.01** | 1.05 | 0.003 | 25.4 | 0.024 | 0.83 | 28.0 | 7.13 | 11.2 | **9.45** | **0.75** | 346 | 5.96 | **33.9** | 15.23 | 16.90 | 331 | 81.0 | 11265 | 1.11 |
| 2 | **18.2** | 8.2 | 340 | 8.1 | 15.91 | 0.010 | 1.03 | 0.005 | 25.1 | 0.027 | 1.00 | 30.6 | 7.10 | 11.6 | 5.58 | **0.75** | 346 | 6.01 | 37.2 | 21.52 | 21.04 | 342 | 93.2 | 13062 | 0.82 |
| 3 | 18.4 | 8.3 | 330 | 9.6 | 15.90 | 0.012 | 1.01 | 0.004 | 25.2 | 0.019 | 0.86 | 30.7 | 7.08 | 11.5 | 2.17 | **0.75** | 343 | 3.86 | 39.2 | 11.07 | 14.89 | 383 | 62.9 | 9930 | 0.56 |
| 4 | 20.9 | **7.7** | 340 | 8.9 | 13.76 | 0.013 | 0.92 | 0.004 | 24.5 | 0.023 | 0.77 | 29.8 | 7.06 | 10.1 | 5.38 | **0.75** | 347 | 6.80 | 54.4 | 16.83 | 19.47 | 318 | 136.0 | 13678 | **3.69** |
| 5 | 20.5 | **7.7** | 350 | 9.8 | 13.37 | 0.013 | 0.88 | 0.002 | 23.6 | 0.012 | 0.81 | 29.9 | 7.05 | **5.0** | 5.08 | **0.75** | 340 | 3.52 | 74.1 | 7.62 | 13.26 | 374 | 42.1 | 6547 | 0.94 |
| 6 | 20.5 | 8.0 | 350 | 9.1 | 14.06 | 0.012 | 0.91 | 0.003 | 24.0 | 0.021 | 0.90 | 28.5 | 7.08 | 10.1 | **1.20** | **0.75** | 346 | 3.67 | 42.5 | 14.21 | 18.35 | 451 | 81.0 | 11501 | 1.54 |
| 7 | 23.0 | 7.8 | 300 | **10.4** | 13.53 | 0.012 | 0.87 | 0.004 | 24.0 | 0.018 | 0.79 | 29.2 | 7.01 | **5.0** | **1.20** | **0.75** | 340 | 2.69 | 45.2 | **5.77** | **7.35** | **570** | 33.4 | 4990 | 0.86 |
| 8 | 20.3 | 7.8 | 330 | 8.6 | 13.57 | 0.007 | 0.93 | 0.007 | 24.0 | **0.030** | 1.12 | 31.4 | 7.13 | **5.0** | **1.20** | **0.75** | 344 | 11.81 | 86.5 | 24.48 | 21.24 | 345 | 95.2 | 13142 | 1.72 |
| 9 | 20.4 | 7.9 | 330 | 8.7 | 13.70 | 0.007 | 0.94 | 0.005 | 23.7 | 0.025 | 0.92 | 31.7 | 7.12 | **5.0** | **1.20** | **0.75** | 344 | 3.47 | 78.6 | 20.74 | 19.31 | 249 | 92.9 | 14127 | 1.87 |
| 10 | 20.9 | 7.8 | 300 | 9.0 | 13.68 | 0.010 | 0.97 | 0.006 | 22.6 | 0.024 | 1.05 | 30.4 | 6.99 | **5.0** | **1.20** | **0.75** | 333 | 3.86 | 82.4 | 21.23 | 19.66 | 259 | 96.7 | **14259** | 1.70 |
| 11 | 20.4 | 7.8 | 390 | 9.2 | **12.20** | 0.011 | 0.88 | 0.006 | **20.8** | 0.022 | 0.96 | **24.7** | **6.24** | **5.0** | **1.20** | **0.75** | 337 | 2.55 | 78.5 | 20.26 | 18.14 | 309 | 79.4 | 12643 | 1.94 |
| 12 | 19.9 | 7.8 | 330 | 8.7 | 13.76 | 0.011 | 0.89 | 0.004 | 22.4 | 0.020 | 1.16 | 28.2 | 7.0 | **5.0** | **1.20** | **0.75** | 331 | 2.20 | 53.3 | 18.19 | 11.36 | 349 | 49.1 | 6922 | 1.76 |
| 13 | 24.5 | 8.0 | 310 | 8.5 | 12.70 | 0.011 | 0.86 | 0.003 | 24.4 | 0.018 | 0.92 | 27.2 | 6.54 | **5.0** | **1.20** | **0.75** | 347 | 2.51 | 74.8 | 9.29 | 17.36 | 293 | 58.4 | 9042 | 1.12 |
| 14 | 21.0 | 8.0 | **230** | **6.0** | 18.05 | 0.011 | 1.04 | 0.017 | 25.9 | **0.006** | 1.35 | 51.7 | 6.85 | 13.5 | 1.35 | 2.95 | 298 | 5.16 | 329.0 | 9.49 | 18.29 | 197 | 61.6 | 10004 | 0.93 |
| 15 | 23.6 | 7.9 | 340 | 7.8 | 14.04 | 0.012 | 0.84 | 0.008 | 24.7 | 0.024 | 1.14 | 30.0 | 6.65 | **5.0** | **1.20** | **0.75** | 346 | 2.35 | 60.7 | 14.40 | 17.74 | 279 | 83.4 | 11066 | 1.53 |
| 16 | 24.2 | 8.4 | 330 | 8.4 | 13.24 | 0.010 | 0.62 | 0.002 | 25.0 | 0.015 | 0.36 | 28.6 | 6.79 | **5.0** | 2.40 | **0.75** | 345 | 6.04 | 89.0 | 13.39 | 17.43 | 176 | 69.4 | 9629 | 1.19 |
| 17 | 25.3 | 8.3 | 330 | 8.4 | 13.76 | 0.008 | **0.39** | 0.005 | 25.7 | 0.018 | **0.26** | 31.3 | 7.04 | **5.0** | **1.20** | **0.75** | 347 | **2.12** | 62.5 | 19.72 | **40.79** | 472 | **155.4** | 6283 | 2.67 |
| 18 | 24.3 | 8.0 | 279 | 6.8 | 18.49 | 0.010 | 1.08 | 0.010 | 27.2 | **0.006** | 1.15 | 54.2 | 6.93 | 14.1 | 1.35 | 2.56 | 318 | 3.04 | 173.9 | 9.56 | 16.61 | 293 | 47.9 | 8377 | 1.99 |
| 19 | 22.3 | 8.1 | 273 | 6.4 | 19.86 | 0.010 | 1.05 | 0.010 | 28.6 | **0.006** | 1.12 | 54.8 | 6.96 | 15.4 | 1.35 | 2.97 | 318 | 2.99 | 117.6 | 11.06 | 22.02 | 194 | 58.0 | 9197 | 0.85 |

| | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 | 25.2 | **9.0** | 288 | 7.2 | 18.69 | 0.007 | 0.77 | **0.001** | 26.7 | 0.018 | 1.32 | 45.1 | 7.36 | 14.7 | 1.35 | 2.85 | 321 | 3.33 | 176.8 | 10.23 | 14.72 | 196 | 39.6 | 6832 | 0.53 |
| 21 | 21.0 | 8.1 | 251 | 6.9 | 23.19 | 0.007 | 1.49 | 0.018 | 28.0 | 0.008 | **2.77** | 35.4 | 6.43 | 21.0 | 4.54 | **3.60** | **213** | **17.58** | **454.5** | **28.59** | 29.63 | **96** | 154.6 | 10394 | 0.32 |
| 22 | 25.4 | 8.0 | 283 | 7.0 | 19.16 | **0.005** | 0.77 | **0.001** | 27.3 | 0.008 | 1.45 | 45.5 | 7.40 | 15.1 | 1.35 | 2.86 | 324 | 3.13 | 165.1 | 6.07 | 11.83 | 415 | **30.7** | **4823** | **0.18** |
| 23 | **26.1** | 8.2 | **531** | 7.4 | **39.00** | 0.010 | **1.55** | **0.024** | **83.6** | 0.012 | 2.06 | **71.7** | **16.3** | **29.8** | 1.35 | 3.17 | **476** | 7.76 | 196.1 | 9.22 | 12.09 | 394 | 41.4 | 5398 | 0.60 |

| Site_No | Density | Size | Eggs | Females_proportion | Juveniles_proportion | Haemocytes | Midgut_gland_dilatation | Kidney_dilatation | Gill_dilatation | Male_pathology | Female_pathology |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 84 | **3,5** | 2 | 44,4 | 73,2 | **0** | 7 | **0** | **0** | **0** | 8 |
| 2 | 25 | 5,3 | 1 | **77,3** | 44,4 | **0** | **0** | **0** | **0** | 20 | 6 |
| 3 | **8** | 5,7 | 3 | 36,4 | 46,7 | **0** | 18 | **0** | **0** | **0** | **0** |
| 4 | 23 | 6,1 | **0** | 38,5 | 18,2 | **0** | 8 | **0** | **0** | **0** | **0** |
| 5 | 152 | **7,5** | **558** | 40,9 | **4,6** | **0** | 5 | **0** | **0** | **0** | **89** |
| 6 | 69 | 6,2 | 27 | 50,0 | 27,6 | 5 | **0** | **0** | **0** | 9 | 18 |
| 7 | 31 | 5 | **0** | 44,4 | 33,3 | **0** | 17 | **0** | **0** | **0** | 25 |
| 8 | 17 | 4,4 | **0** | 52,4 | 61,1 | 5 | 9 | **0** | **0** | **0** | 46 |
| 9 | 65 | 4,1 | **0** | 45,5 | 65,5 | **0** | **0** | **0** | **0** | **0** | 10 |
| 10 | 41 | 5,7 | 2 | 57,9 | 28,2 | 5 | **0** | **0** | **0** | **0** | **0** |
| 11 | **546** | **3,5** | 10 | 52,6 | **78,3** | 5 | 21 | **0** | **0** | **0** | 20 |
| 12 | 118 | 5,1 | 34 | 31,8 | 50,0 | **0** | 9 | **0** | **0** | **0** | **0** |
| 13 | 163 | 3,7 | 116 | 75,0 | 72,3 | **25** | 5 | 5 | **0** | **0** | 33 |
| 14 | 33 | 6,3 | 1 | 52,9 | 18,2 | **0** | 38 | 4,2 | **16,7** | **0** | **0** |

| Site_No | Density | Size | Eggs | Females_proportion | Juveniles_proportion | Haemocytes | Midgut_gland_dilatation | Kidney_dilatation | Gill_dilatation | Male_pathology | Female_pathology |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | 13 | 5,1 | **0** | 33,3 | 50,0 | **0** | 13 | **0** | **0** | **0** | 13 |
| 16 | 13 | 6 | 3 | 37,5 | 33,3 | 6 | **50** | **0** | **0** | **0** | 50 |
| 17 | 51 | 5,4 | 49 | **16,7** | 58,5 | **0** | 22 | **0** | **0** | **0** | 67 |
| 18 | 181 | 6,1 | 102 | 38,5 | 17,6 | **0** | 8 | **0** | **0** | 13 | **0** |
| 19 | 96 | 5,8 | 32 | 33,3 | 25,5 | 2 | 15 | **6,5** | **0** | **0** | **0** |
| 20 | 289 | 4,7 | 294 | 35,7 | 66,9 | **0** | **0** | **0** | **0** | 22 | 60 |
| 21 | 138 | 5,6 | 39 | 60,0 | 36,7 | **0** | **0** | **0** | **0** | **25** | 17 |
| 22 | 207 | 5,7 | 235 | 33,3 | 25,8 | **0** | **0** | **0** | **0** | 15 | 10 |
| 23 | 152 | 4,2 | 1 | 27,3 | 55,3 | **0** | **0** | **0** | **0** | 13 | 33 |

# Paper 3

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PRIMARY RESEARCH PAPER

# The Ponto-Caspian parasite *Plagioporus* cf. *skrjabini* reaches the River Rhine system in Central Europe: higher infestation in the native than in the introduced Danubian form of the gastropod *Theodoxus fluviatilis*

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**Abstract** The introduction of non-indigenous organisms in new areas in the context of host-parasite interactions is still poorly understood. This study aimed at a parasitological and histopathological comparison of two phylogenetically distinct forms of the freshwater snail *Theodoxus fluviatilis* in the River Rhine system: the native Northern-European form, which showed a decline for unknown reasons and is nowadays extinct in the River Rhine, and the non-indigenous Danubian form, which was introduced via the Main–Danube canal. We histopathologically examined populations of Northern-European *T. fluviatilis* from three smaller rivers of the Rhine system and of Danubian *T. fluviatilis* from the River Rhine, after confirming the phylogenetic background of the respective population genetically. Results showed differences in the prevalence of trematodes and histopathologic organic alterations between the two snail forms. Both were infected with an opecoelid trematode *Plagioporus* cf. *skrjabini*, whereby its prevalence was significantly higher in the Northern-European than in the Danubian form. The parasitic trematode is, to our knowledge, a new trematode species in the River Rhine system, presumably co-introduced through the invasion of its second intermediate and final hosts, i.e. Ponto-Caspian amphipods and gobies. Its impact on native populations of Northern-European *T. fluviatilis* needs to be subject of future studies.

**Keywords** Trematoda · Cercariae · Prevalence · Main–Danube canal · *Dikerogammarus villosus* · *Neogobius*

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## Introduction

The aquatic snail *Theodoxus fluviatilis* (Linné, 1758) (Gastropoda: Neritidae) is a characteristic species in freshwater ecosystems and has often been used as an indicator for river assessment and monitoring (e.g. Zettler, 2008). It is the most widespread species of the genus *Theodoxus* in Central Europe and native to all large rivers in Germany. With regard to the River Rhine and its tributaries, a decrease in the spatial distribution of *T. fluviatilis* is documented for the last decades, and nowadays only relict populations can be found in smaller tributaries of the River Rhine and the River Main system in Southwestern and Central Germany (Zettler, 2008). In the main stretch of the River Rhine, *T. fluviatilis* disappeared in the late 1990s for unknown reasons, until 2006, when morphologically different individuals were recorded in the River Rhine near the mouth of the River Main (Westermann et al., 2007). Genetic analysis of these specimens and comparisons with genetic material of native River Rhine populations revealed that the newly discovered snails phylogenetically belong to the Danubian form of *T. fluviatilis*, which is known from the Danube and Black Sea drainages (Bunje, 2005; Gergs et al., 2015). It is assumed that this originally Ponto-Caspian form of *T. fluviatilis* was introduced to the River Rhine by shipping through the Main–Danube canal that was opened in the year 1992 (Gergs et al., 2015). The Main–Danube canal connects the Danube to the River Rhine via the River Main and enables the spread of numerous non-indigenous species originating from the Ponto-Caspian system via the so-called southern corridor, e.g. by transport through hull fouling and ballast water of ships or active migration (e.g. Bij de Vaate et al., 2002). Currently, the Danubian form of *T. fluviatilis* in the River Rhine is observed to spread along the river since its discovery (IKSR/CIPR/ICBR, 2012) and to establish high population densities even in anthropogenically degraded habitats like harbours (Rothmeier & Martens, 2019).

The increasing occurrence of non-indigenous species in freshwater ecosystems in Central Europe, due to the spreading of Ponto-Caspian alien species, leads to various changes in ecological interactions including host-parasite relationships (e.g. Emde et al., 2014). For example, invasive North American crayfish species in Europe carry the parasitic oomycete *Aphanomyces astaci* Schikora, 1906, which is the causative agent of the crayfish plague affecting native crayfish populations (e.g. Söderhäll & Cerenius, 1999; Holdich et al., 2009). The co-introduction of generalist parasite species with low host specificity is observed to be important in invasions and can lead to their establishment if they are able to complete their life cycle in native hosts within the invaded area (Prenter et al., 2004; Alt et al., 2019). One of the better recognised groups of endoparasites in molluscan hosts are trematodes characterised by a complex life cycle (e.g. Cichy et al., 2011; Emde et al., 2014). Molluscs play an obligatory role of the first intermediate hosts for both the proliferation (e.g. sporocysts) and transmission (cercariae) stages (Esch et al., 2002). Trematodes affect the morphology, physiology, and behaviour of snails, which increases the chance of transmission to the next host (Herbison et al., 2018).

The influence of biological invasions on interspecies interactions, including parasitism, is still insufficiently recognised. Therefore, the aim of this study is a parasitological and histopathological comparison of populations of the newly introduced Danubian form and the native Northern-European form of *T. fluviatilis* in different rivers of the Rhine system in Central Europe, after confirming the phylogenetic difference of the respective population genetically. By analysing specimens from sites at the River Rhine on one hand and from three smaller rivers belonging to the River Rhine system (i.e. Jagst, Kocher and Tauber) on the other hand, we focus on detecting differences regarding infestations of trematodes and histopathological organic alterations.

## Materials and methods

### Sampling of *Theodoxus fluviatilis* and parasites

Sampling of individuals of *T. fluviatilis* was done in August and September 2018 at seven sites along the River Rhine, from Neuburg at river-km 355 to Mannheim at river-km 430, as well as at one site of the river Jagst, three sites of the river Kocher, and three sites of the river Tauber, i.e. a total of 14 sampling sites (Table 1). Two subsamples of snails were collected manually from riprap stones: 5 animals per sampling site were stored in pure 96% ethanol for genetic analysis (overall 70 individuals), and 12 to 46 snails

**Table 1** Sampling sites, dates, the respective haplotypes, accession numbers and allocation to phylogenetic groups of *Theodoxus fluviatilis* individuals along the River Rhine and the rivers Jagst, Kocher and Tauber

| River | Site name | Coordinate (WGS84) | Sampling date (month/year) | Haplotype | Accession number | Phylogenetic group |
|---|---|---|---|---|---|---|
| Rhine | Neuburg | 48°59′12″N, 8°16′10″E | 09/2018 | F31/F34 | AY765336/AY765341 | Danubian[b] |
| | Karlsruhe | 49°02′07″N, 8°18′16″E | 09/2018 | F31 | AY765336 | |
| | Eggenstein | 49°05′24″N, 8°21′13″E | 09/2018 | F31 | AY765336 | |
| | Leimersheim | 49°07′17″N, 8°21′50″E | 08/2018 | F31/F34 | AY765336/AY765341 | |
| | Speyer | 49°19′10″N, 8°26′59″E | 08/2018 | F31 | AY765336 | |
| | Altrip | 49°26′03″N, 8°30′25″E | 08/2018 | F31/new[a] | AY765336/MT563453[a] | |
| | Mannheim | 49°32′37″N, 8°25′26″E | 08/2018 | F31 | AY765336 | |
| Jagst | Herbolzheim | 49°16′59″N, 9°15′31″E | 09/2018 | "Tauber" | MG969538 | Northern-European |
| Kocher | Ingelfingen | 49°17′53″N, 9°39′09″E | 09/2018 | F1 | AY765306 | |
| | Ernsbach | 49°17′26″N, 9°30′53″E | 09/2018 | F1 | AY765306 | |
| | Kochendorf | 49°13′39″N, 9°13′03″E | 09/2018 | F1 | AY765306 | |
| Tauber | Igersheim | 49°29′30″N, 9°48′46″E | 09/2018 | "Tauber" | MG969538 | |
| | Lauda | 49°34′14″N, 9°42′28″E | 09/2018 | "Tauber" | MG969538 | |
| | Bronnbach | 49°41′56″N, 9°32′54″E | 09/2018 | "Tauber" | MG969538 | |

[a]New haplotype from the present study

[b]Three analysed individuals harboured the haplotype F34 belonging to the "*Theodoxus* cf. *velox*"-group (Bunje, 2005), nevertheless they are summarised as Danubian together with those of the haplotype F31 in the present study (for detailed information see results)

per sampling site were transported to the lab in river water for histopathological analysis, whereby varying numbers of individuals per sampling site resulted from the availability of specimens with at least 5 mm shell length to conduct histological preparation. Overall, 212 specimens from the River Rhine and 150 specimens from the rivers Jagst, Kocher and Tauber, i.e. in total 362 individuals, were examined histopathologically. According to the Regional Council Karlsruhe, Baden-Württemberg, a sampling permission was not needed as *T. fluviatilis* is not a specially protected species according to the Federal Nature Conservation Act of Germany.

For identification of parasites found in *T. fluviatilis*, ten adult snails (shell length 9 ± 1 mm) from one site of the Kocher near Ingelfingen (Table 1; selection of this site due to the high trematode prevalence (90%), see results) were stored in pure 96% ethanol, dissected under an EZ4 microscope (Leica, Germany) and checked for parasites. Sporocysts and cercariae which were found in the visceral bag of the snails were mounted on glass slides, using a DM500 microscope (Leica, Germany) for morphological identification of the parasite species, which was carried out on the basis of morphological features of opecoelid cercariae (e.g. Chernogorenko et al., 1978; Cribb, 2002). Three subsamples of cercariae including approximately 20 specimens were stored in pure 96% ethanol for genetic analysis.

## Genetic analysis of snails and trematodes

For genetic identification of the snails, animals were excerpted from their shell and muscle tissue was cut from the foot (slices of 1-2 mm) for DNA extraction using a modified high salt-extraction protocol (Koester & Gergs, 2017, 2014). Sequences of the mitochondrial cytochrome c oxidase subunit I gene (COI) were amplified by polymerase chain reaction (PCR) using the primers F4d (5′-TACTTTRTATATTATGTTTGGT-3′), and R1d (5′-TGRTAWARAATDGGRTCWCCHCCVCC-3′) (Bunje, 2005). Each 40 µl PCR reaction mixture contained 1 × reaction buffer S, 0.3 mM dNTPs, 2.75 mM $MgCl_2$ (Peqlab Biotechnologie, Germany), 0.05 U Taq DNA Polymerase (Peqlab Biotechnologie, Germany), 0.5 µM of each primer (Eurofins Genomics, Germany), and approximately 4 ng genomic DNA of a single individual. The following PCR protocol was used to amplify the gene region: 95°C for 10 min,

followed by 36 cycles of 95°C for 50 s, 54°C for 60 s, 72°C for 60 s, and a final extension at 72°C for 7 min. PCR products were sequenced by the company SeqIT (Kaiserslautern, Germany) in forward and reverse direction using a 3730 DNA Analyzer eight capillary sequencer (AppliedBiosystems, USA). We used Geneious 11.1.2 (Biomatters Ltd, available from www.geneious.com) to manually edit and align all sequences with known unique haplotypes of *T. fluviatilis* (GenBank accession nos. AY765306-AY765345, KJ493817, and MG969538; Bunje, 2005; Gergs et al., 2015; Richling & Groh, 2018) and one sequence of *Theodoxus danubialis* (Pfeiffer, 1828) (GenBank accession no. AY771303; Bunje, 2005).

Samples of cercariae ($n$ = 3) found in *T. fluviatilis* collected from the river Kocher were genetically analysed by the company MBS Szkolenia Konferencje Usługi (Warsaw, Poland). Genomic DNA was extracted using a NucleoSpin Tissue Kit (Macherey–Nagel, Germany), following the manufacturer's instructions (according to Behrens-Chapuis et al., 2018). DNA was eluted in 50 µl of elution buffer and stored at -20°C. Amplification of the COI gene was carried out using the primers Dice1F (5′-ATTAACCCTCACTAAATTWCNTTRGATCA-TAAG-3′) and Dice11R (5′-TAATACGACTCAC-TATAGCWGWACHAAATTTHCGATC-3′) (van Steenkiste et al., 2015). Each 40 µl PCR reaction mixtures contained 20 µl RedTaq ReadyMix (Sigma-Aldrich, Germany), 4 µl primer mix (concentration of each primer 5 µM), 2 µl DNA, and 16 µl double-distilled water (< 18.2 MΩ). The PCR profile included an initial denaturation at 94°C for 2 min, followed by 3 cycles of 94°C for 40 s, 51°C for 40 s, 72°C for 60 s; 5 touchdown cycles of 94°C for 40 s, 50°C to 46°C for 40 s (decreasing 1°C per cycle), 72°C for 60 s; 35 cycles of 94°C for 40 s, 45°C for 40 s, 72°C for 60 s, and a final extension at 72°C for 300 s (van Steenkiste et al., 2015). Sequencing was done using a 3500xl sequencer (AppliedBiosystems, USA) in forward and reverse direction. Sequences ($n$ = 3) were edited and aligned using de novo assembly in the CAP3 program (Huang & Madan, 1999), resulting in a contig sequence. Species affiliation was determined using BOLDSYSTEM (available from www.boldsystems.org) and BLAST (National Center for Biotechnology Information, available from https://blast.ncbi.nlm.nih.gov/Blast.cgi).

### Parasitological and histopathological analysis

To ensure complete defaecation, snails collected for histopathological analysis were kept in river water in the laboratory for 3 days and were subsequently histologically prepared following the protocol of Watermann et al. (2008). Individuals were narcotized in magnesium chloride (3%) before fixation in Bouin's fluid for 16 days, then washed and stored in 80% ethanol (p.a.). Afterwards, snails were embedded in paraffin and cut into 2–3 µm sections with a rotation microtome (Microm, Walldorf, Germany). Sections were mounted on glass slides and counter-stained with haematoxylin and eosin (e.g. Mulisch & Welsch, 2010). According to Rothmeier et al. (2020), the following snail's tissues and organs were checked for the presence of trematodes and histopathological alterations: foot tissue, digestive gland, stomach, kidney, gill, and sexual organs (in females: gonads, receptaculum seminis, and accessory glands; in males: gonads, vesiculum seminis, and prostate gland). Alterations considered as pathologic were tissue dilatation or degeneration, black or brown pigment depositions, e.g. in stomach and digestive gland, and inhibited or arrested spermatogenesis or oogenesis in gonads. For further calculations, the percentage of affected snails per sampling site was calculated for each detected alteration. Trematode prevalence (% of infected snails) per sampling site was calculated according to Bush et al. (1997), i.e. the number of hosts infected with one or more individuals of a particular parasite species, i.e. miracidium, sporocyst or cercaria, was divided by the number of snail hosts examined. Sex-specific histopathological findings in male and female gonads were summed up as pathological findings in sexual organs.

### Statistical analysis

All statistical analyses were done using the statistical software package R (version 3.6.3, R Core Team, 2020). Phylogenetic analysis of DNA-sequences of *T. fluviatilis* and cercariae and building of maximum likelihood (ML) trees was conducted using a ML analysis with the package *phangorn* (Schliep, 2011) according to the protocol of Gergs et al. (2015). For ML analysis, the substitution model TPM3u + I was determined for both, *T. fluviatilis* and cercariae, using jModelTest and the Akaike information criterion

(Posada, 2008). By using the *pml* function of the package *phangorn*, the likelihood of a phylogenetic tree was computed and optimised with *optim.pml*. Bootstrapping was conducted with 1000 replicates to estimate the support for reconstructed branches of the ML tree.

To analyse differences in trematode prevalence and histopathological alterations between the two phylogenetically distinct snail forms (Danubian and Northern-European, see Results and Table 1), a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted using the *adonis* function of the R package *vegan* (version 2.5-7, Oksanen et al., 2020). Arcsine and square root transformed proportional data of trematode prevalence together with six histopathological findings (digestive gland dilatations, pigment depositions in digestive glands and in stomachs, gill dilatations, kidney dilatations, and pathological findings in sexual organs) were compared between the Danubian and Northern-European form using Bray–Curtis dissimilarity as a distance measure (999 permutations) and sampling sites as replicates. The percentual contributions of single parameters to overall dissimilarity were analysed by calculating similarity percentages using *vegan's* function *simper*. The Mann–Whitney-*U*-test for independent samples was used to conduct a univariate comparison of trematode prevalence between the two phylogenetically distinct forms of *T. fluviatilis*.

## Results

### Genetic analysis of snails and trematodes

At the rivers Jagst and Tauber, all sampled individuals of *T. fluviatilis* were identified as the "Tauber"-haplotype (acc. no. MG969538; Table 1), and snails sampled from the three sites of the river Kocher belonged to the haplotype F1 (acc. no. AY765306; Table 1). Both the haplotypes "Tauber" and F1 are descendants from the basal haplotype F3, phylogenetically belonging to the Northern-European group of *T. fluviatilis* (Fig. 1). 31 out of 35 *T. fluviatilis* specimens analysed from the seven sampling sites of the River Rhine harbour the haplotype F31 (acc. no. AY765336; Table 1), belonging to the Danubian group (Fig. 1). Out of each five individuals per sampling site, one individual of the site near Neuburg and two individuals of the site near Leimersheim were identified as haplotype F34 (acc. no. AY765341; Table 1), which is assigned to the Ponto-Caspian "*Theodoxus* cf. *velox*"-group (Fig. 1). Additionally, one new haplotype (acc. no. MT563453) was found in a single individual from the River Rhine at the site near Altrip (Table 1). Based on the ML analysis for phylogenetic reconstruction, the newly described haplotype MT563453 also belongs to the Danubian group (Fig. 1). Resulting from genetic analyses, snails sampled for this study can be divided into two phylogenetically distinct forms: (i), individuals from sampling sites of the River Rhine are summarised as Danubian form, knowing that this group also harbours three presumably co-introduced individuals of the "*Theodoxus* cf. *velox*"-group from the Ponto-Caspian region, and (ii), snails from the rivers Jagst, Kocher and Tauber can be summarised and allocated to the Northern-European form of *T. fluviatilis* (Fig. 1).

Genetic analyses of trematodes showed that the species found in *T. fluviatilis* represented the genus *Plagioporus* (Allocreadiata, Opecoelidae). The obtained COI gene sequence from cercariae (acc. no. MT756015.1; this study) showed 81% similarity to *Plagioporus sinitsini* Mueller, 1934 (acc. no. KM538106; van Steenkiste et al., 2015) and 76% similarity to *Echinostoma revolutum* (Fröhlich, 1802) (acc. no. NC_046395; Ran et al., 2020) (Fig. S1). Morphological features of cercariae correspond to the species *Plagioporus skrjabini* Kowal, 1951 (Fig. 2). Due to the morphological similarity to *P. skrjabini* and the genetic affiliation to the genus *Plagioporus*, but the lack of COI sequences in GenBank, the name *Plagioporus* cf. *skrjabini* will be used to emphasize uncertain species affiliation.

### Parasitological and histopathological analysis

Multivariate statistical analysis revealed a significant difference in the prevalence of *P.* cf. *skrjabini*, and histopathological parameters (digestive gland, gill and kidney dilatations, pigment depositions in digestive gland and stomachs and pathological findings in sexual organs) between the Northern-European and the Danubian form of *T. fluviatilis* (PERMANOVA, $P = 0.02$; see Fig. 3 for biplot of dissimilarity matrix based on Bray–Curtis dissimilarity). Of the analysed parameters, the prevalence of *P.* cf. *skrjabini* showed

the highest contribution to overall dissimilarity between the two snail forms (*simper*, 28.7%; for percentual contributions of other variables see Table S1). The prevalence of *P.* cf. *skrjabini* was significantly higher in the Northern-European (52.5 ± 35.5%) than in the Danubian form (3.6 ± 3.5%) of analysed *T. fluviatilis* (Mann–Whitney-*U*-test, $P = 0.004$; Fig. 4).

Different larval stages of *P.* cf. *skrjabini* were found in parasitised *T. fluviatilis*, i.e. miracidia, sporocysts and cercariae, which were located in various organs of the snails. At an early infestation stage, penetrating miracidia were found in the foot of the snails (Fig. S2a), from where they spread into the stomach developing sporocysts (Fig. S2b) and finally infect the digestive gland tissue (Fig. S2c) and gonads of the snails (Fig. S2d). Digestive glands and gonads of heavily parasitised snails were observed to be full of sporocysts (Fig. S2e), in which cercariae were in a developing stage or already mature and released into the host's tissue, leaving extensive lacunas (Fig. S2e, f).

## Discussion

Comparison of the native Northern-European and the newly introduced Danubian form of *T. fluviatilis* in Central Europe showed significant differences in the prevalence of *P.* cf. *skrjabini* and histopathological organic alterations. Though the parasite was found in both snail forms, it had a significantly higher prevalence in the Northern-European form of *T. fluviatilis*. To our knowledge, this study provides the first record of a parasitic trematode of the genus *Plagioporus*, most likely *P. skrjabini*, in the Northern-European form of *T. fluviatilis*. *P. skrjabini* has previously been described in the Ponto-Caspian region from *T. fluviatilis* being the first intermediate host, and from other hosts, including amphipods and predatory fish like gobies (Chernogorenko et al., 1978). Presumably, the trematode could have been introduced to the River Rhine system through the invasion of its second intermediate or final hosts, i.e. amphipods as *Dikerogammarus villosus* (Sowinsky, 1894) or Ponto-Caspian gobies, via the southern corridor. *D. villosus* has been discovered in the lower reaches of the River Rhine in 1994 (Bij de Vaate & Klink, 1995) and is nowadays the predominant amphipod species in the river (Alt et al., 2019). The bighead goby, *Neogobius kessleri* (Günther, 1861), and the monkey goby, *Neogobius fluviatilis* (Pallas, 1814), occur in the River Rhine since 2007 and 2009, respectively (van Kessel et al., 2009). The results of the present study resemble the findings of Hohenadler et al. (2018), who found that the Ponto-Caspian acanthocephalan parasite *Pomphorhynchus laevis* (Zoega in Müller, 1776) was recently co-introduced to European waterbodies together with different host species such as *D. villosus* and *Neogobius melanostomus* (Pallas, 1814). The introduction of *P.* cf. *skrjabini* into the River Rhine and its tributaries could be another example of a host-parasite system which originates from the Ponto-Caspian region.

The mechanism of the introduction of new parasite species to local hosts through an invasive species, and the resulting increase of prevalence in native host communities is called parasite spillover (e.g. Hohenadler et al., 2019). It has already been observed for example by an examination of the marine parasitic copepod *Mytilicola orientalis* Mori, 1935, in Northern Europe, which does not only infect its principal host, the invasive Pacific oyster *Crassostrea gigas* (Thunberg, 1793), but also native blue mussels *Mytilus edulis* Linné, 1758 and common cockles *Cerastoderma edule* (Linné, 1758) (Goedknegt et al., 2017). Hence, it is probable that a parasite spillover of *P.* cf. *skrjabini* from its Ponto-Caspian invasive hosts, e.g. amphipods and/or gobies, to the Northern-European *T. fluviatilis* occurred in the River Rhine system. Whilst the Danubian form potentially experienced some kind of co-evolution due to the already occurred infestation with *P. skrjabini* in its native range (Chernogorenko et al., 1978), the Northern European form may represent a new host for the parasitic trematode in its introduced region of the River Rhine and its tributaries. Histopathological findings in the snail's organs can be associated with trematode infection as well as environmental stressors at the sampling sites. Trematode-associated effects on reproductive organs of infected snails are well studied, as parasites use these resource-rich tissues to grow and develop within hosts (reviewed by Sorensen & Minchella, 2001). Increased depositions of lipopigments and dilatations in epithelial cells and tubules of the mollusc's organs, e.g. the digestive system can be associated with unspecific immune reactions (Watermann et al., 2008), either towards parasite

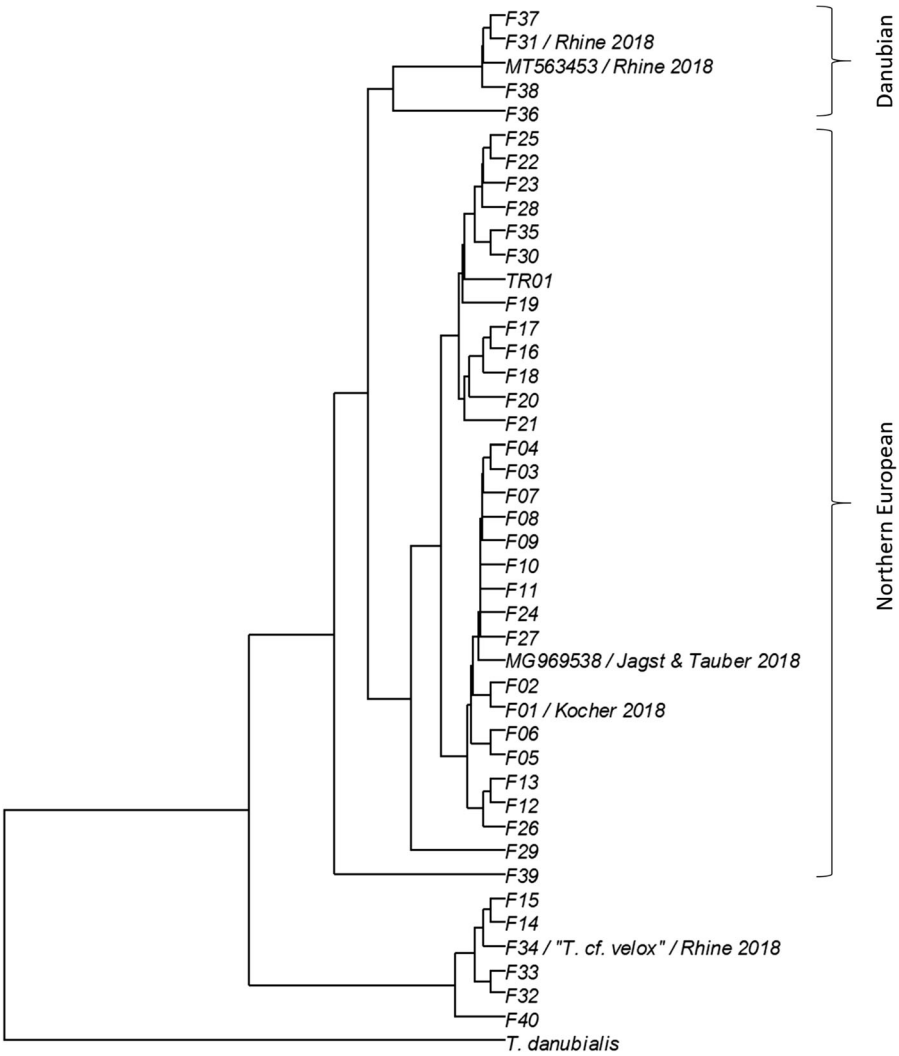


**Fig. 1** Maximum likelihood tree of unique haplotypes of *Theodoxus fluviatilis*, rooted with an outgroup haplotype (*T. danubialis*). Only bootstrap values ($n = 1000$) of the main branches are displayed. For geographical classification of the haplotypes found in 2018 in the River Rhine and the rivers Jagst, Kocher and Tauber see Table 1. See Bunje (2005), Gergs et al. (2015), and Richling and Groh (2018) for further information about haplotypes, classification groups and phylogeographic clades of *T. fluviatilis*

infections or environmental toxicants. However, which factors are responsible for the high prevalence and which consequences may occur on the population level of infected native Northern-European *T. fluviatilis* are unknown so far.

As parasites have often been demonstrated as useful indicators of environmental pollution (reviewed by Sures et al., 2017), the high prevalence of the trematode *Plagioporus* cf. *skrjabini* in Northern-European *T. fluviatilis* might be fostered by environmental stressors. The rivers Jagst, Kocher and Tauber are characterised by moderate pollution (water quality class II; LAWA, 2002) and high species-richness regarding macroinvertebrates (unpublished data). If the difference in the prevalence between the two forms of *T. fluviatilis* is caused by environmental stressors, different susceptibility to parasite pressure or a combination of both factors needs to be addressed in future studies.

In conclusion, this study shows for the first time that native populations of the Northern-European form of *T. fluviatilis* have been infected by the Ponto-Caspian

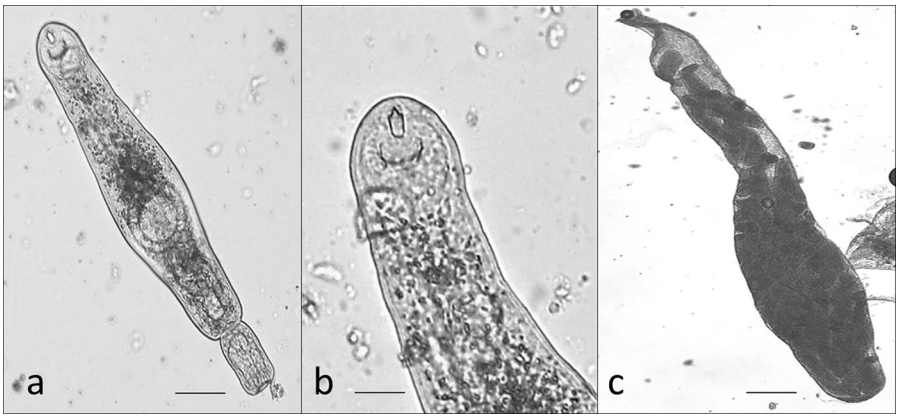


**Fig. 2** Microscope photographs of different life cycle stages of *Plagioporus* cf. *skrjabini* from the visceral bag of the freshwater snail *Theodoxus fluviatilis* (sampled at the river Kocher, Germany). **a** Cercaria of *P.* cf. *skrjabini*. Bar 50 µm. **b** Stylet of *P.* cf. *skrjabini* cercaria. Bar 25 µm. **c** Sporocyst of *P.* cf. *skrjabini* filled with cercariae. Bar 300 µm

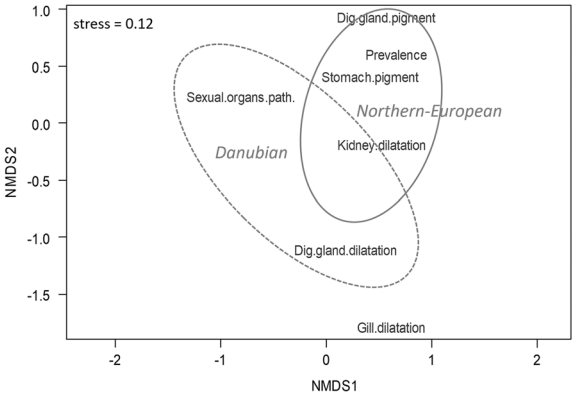


**Fig. 3** Nonmetric Multidimensional Scaling (NMDS) biplot of the dissimilarity matrix (Bray–Curtis dissimilarity) of *Plagioporus* cf. *skrjabini* prevalence and histopathological alterations in two phylogenetic distinct forms of the snail *Theodoxus fluviatilis* from sampling sites at the River Rhine (Danubian form, $n = 7$) and the rivers Jagst, Kocher and Tauber (Northern European form, $n = 7$). Prevalence and histopathological variables were added using weighted averages. Site scores are not shown for clarity. Dig.gland.dilatation = pathologic dilatation of digestive gland tissue, Dig.gland.pigment = black or brown pigment depositions in digestive gland tissue, Gill.dilatation = pathologic dilatation of gill tissue, Kidney.dilatation = pathologic dilatation of kidney tissue, Prevalence = prevalence of trematode *P.* cf. *skrjabini* in *T. fluviatilis*, Sexual.organs.path = pathologic alterations in male or female gonads, Stomach.pigment = black or brown pigment depositions in stomach tissue

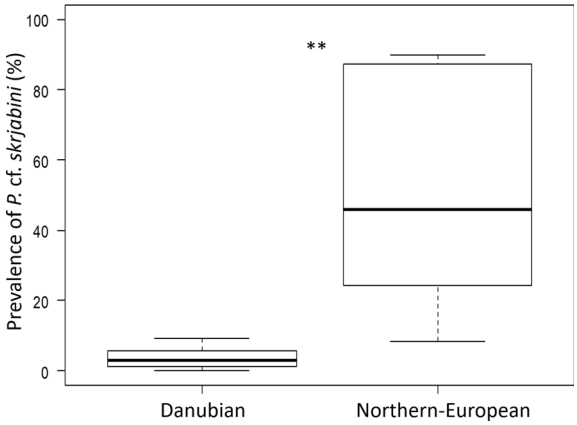


**Fig. 4** Prevalence (%) of the parasitic trematode *Plagioporus* cf. *skrjabini* in two phylogenetic distinct forms of the snail *Theodoxus fluviatilis* at the River Rhine (Danubian form, $n = 7$) and the rivers Jagst, Kocher and Tauber (Northern-European form, $n = 7$). Asterisks indicate statistically significant difference (Mann–Whitney-*U*-test, $P = 0.004$)

parasitic trematode *P.* cf. *skrjabini*. Presumably, the trematode has been co-introduced to the Upper River Rhine system through the invasion of its second intermediate and final hosts (Ponto-Caspian amphipods and gobies). The presence of both the non-indigenous species of parasites and hosts outside their natural ranges can have an essential impact on native biota. As the effects of interactions are difficult to estimate, emphasis should be placed on monitoring the condition of native populations such as the Northern-European form of *T. fluviatilis*.

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**Data availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflicts of interest** The authors declare that they have no conflict of interest and no competing interests.

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Supplementary material to

# The Ponto-Caspian parasite *Plagioporus* cf. *skrjabini* reaches the River Rhine system in Central Europe: higher infestation in the native than in the introduced Danubian form of the gastropod *Theodoxus fluviatilis*

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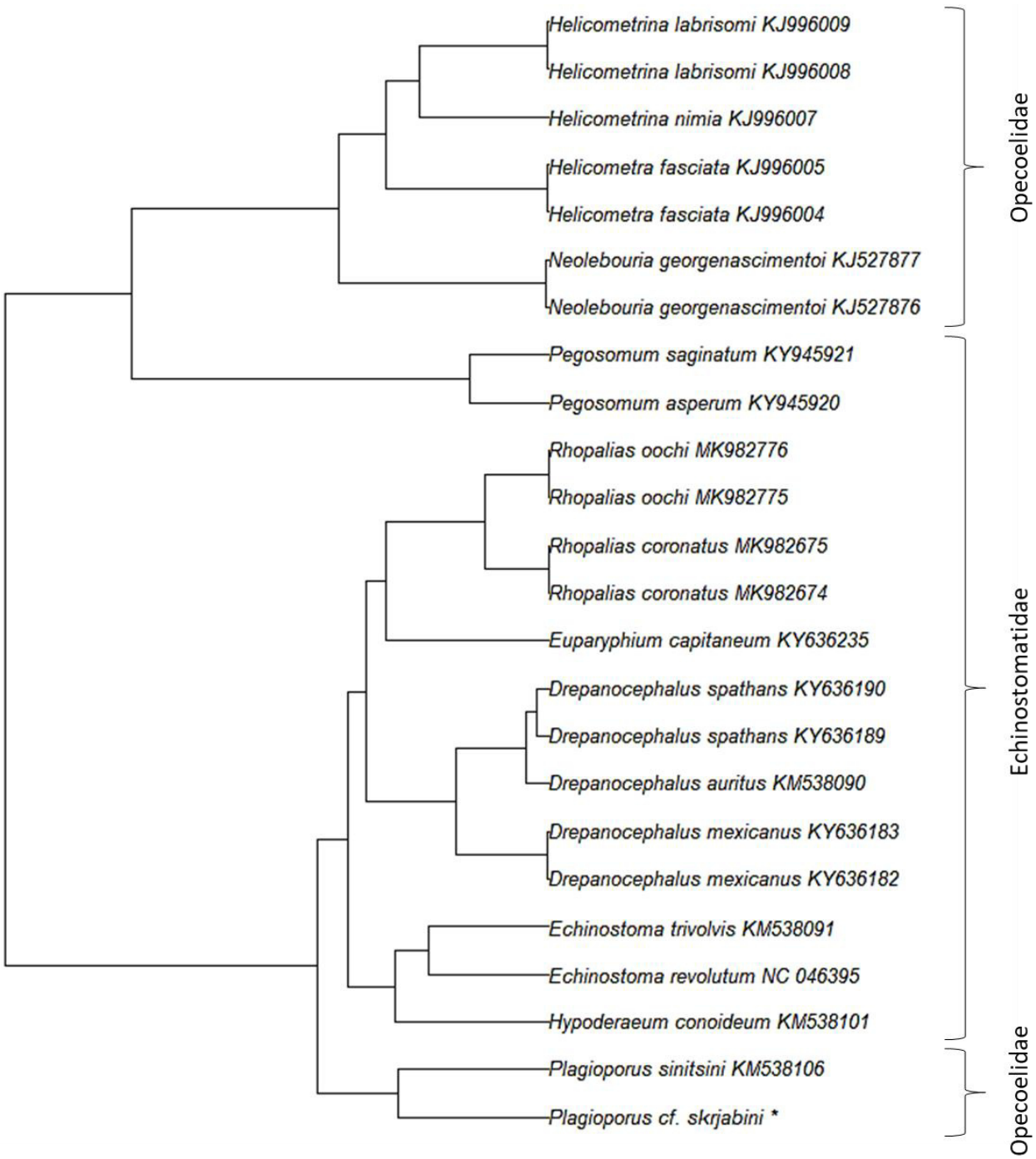


**Figure S1** Maximum likelihood tree of *Plagioporus* cf. *skrjabini* cercariae (marked by an asterisk; GenBank acc. no. MT756015.1, this study) found in the freshwater snail *Theodoxus fluviatilis* and 23 GenBank COI sequences from trematode species belonging to the Echinostomatidae and Opecoelidae families (acc. nos. displayed). Only bootstrap values (n = 1,000) of the main branches are displayed.

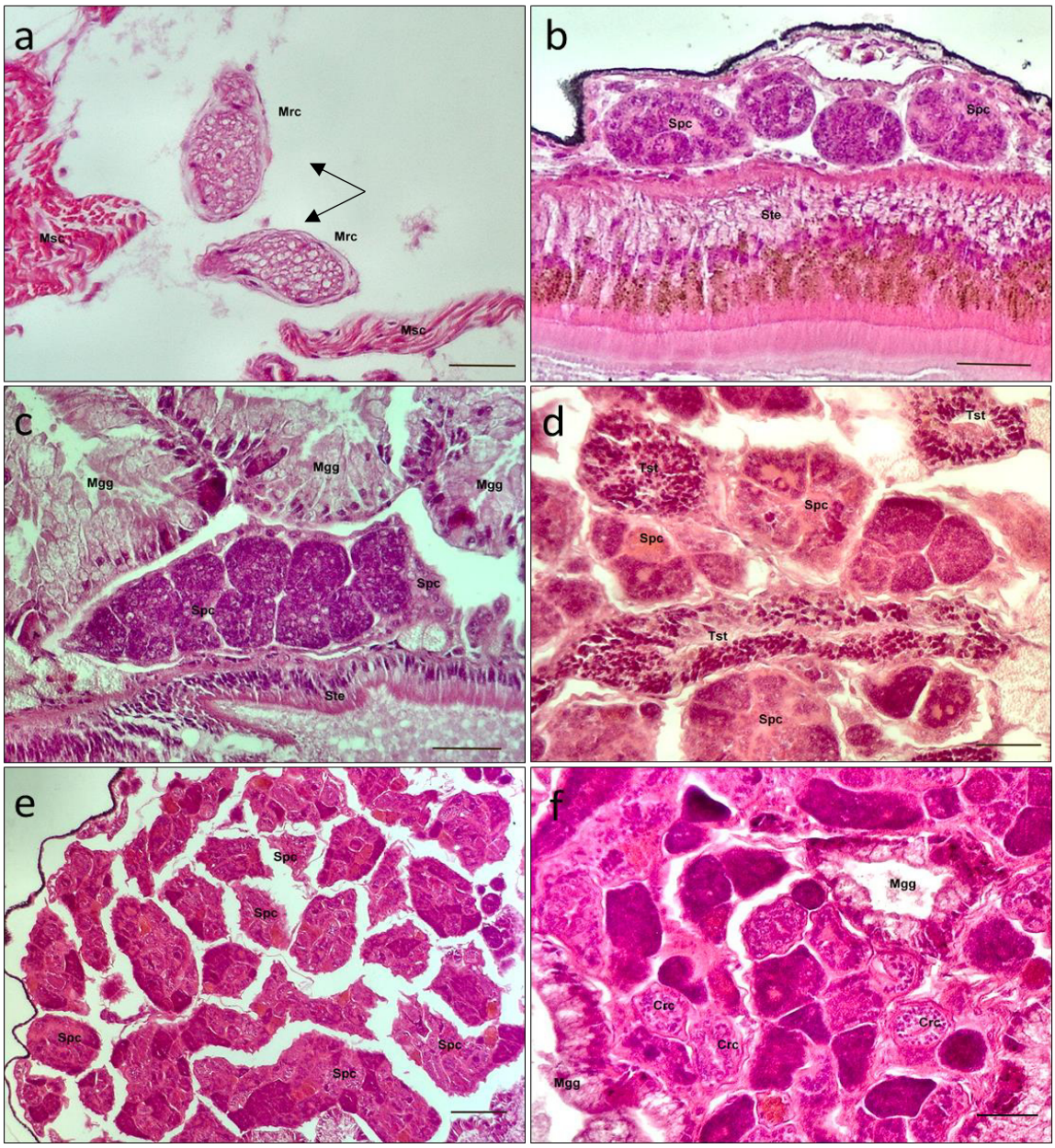


**Figure S2** Microscope photographs of histologically prepared sections (2-3 µm) from tissues of the Northern-European form of *Theodoxus fluviatilis*. Counter-staining with haematoxylin and eosin, bar 50 µm. Crc = cercaria, Mgg = midgut (digestive) gland, Mrc = miracidium, Msc = muscle, Spc = sporocyst, Ste = stomach epithelium, Tst = testis; **a** foot with *Plagioporus* cf. *skrjabini* miracidia between separated muscle bundles (arrows indicating cilia); **b** stomach epithelium with brown pigment deposition and sporocysts of *P.* cf. *skrjabini*; **c** digestive gland tubuli with sporocysts of *P.* cf. *skrjabini*; **d** and **e** gonad tissue with intensive *P.* cf. *skrjabini* infestation (sporocysts) and lacunas; **f** digestive gland tubuli with released cercariae of *P.* cf. *skrjabini*.

**Table S1** Results of *similarity percentages (simper)* analysis of percentual contributions of *Plagioporus* cf. *skrjabini* prevalence and six histopathological parameters to overall Bray-Curtis dissimilarity of Permutational Multivariate Analysis of Variance (PERMANOVA, 999 permutations) comparing two phylogenetically distinct forms of *Theodoxus fluviatilis* (Danubian and Northern-European).

| Parameter | Contribution to overall Bray-Curtis dissimilarity (%) |
|---|---|
| Prevalence of *Plagioporus* cf. *skrjabini* | 28.7 |
| Sexual organs pathological findings | 20.4 |
| Stomach pigment deposition | 18.7 |
| Digestive gland pigment deposition | 16.4 |
| Digestive gland dilatation | 8.3 |
| Kidney dilatation | 5.9 |
| Gill dilatation | 1.7 |

# Paper 4

# Evaluation of a preliminary test design for a growth experiment with juveniles of two phylogenetic different forms of the freshwater snail *Theodoxus fluviatilis*

Louisa Marie Rothmeier[1], Andreas Martens[2], Burkard Watermann[3], René Sahm[4]

**Abstract** The progressive contamination of aquatic environments poses multiple challenges towards ecological risk assessment, e.g. the need of reliable and suitable ecotoxicological test strategies and organisms. However, the intraspecific variability of indicator species considering differences in sensitivity towards environmental pollutants is still poorly recognised, though being biological reality. We aimed at doing a first step towards examining the intraspecific variability between two phylogenetically different forms of the freshwater snail *Theodoxus fluviatilis* in German inland waters, the native Northern-European and the introduced Danubian form. A preliminary test design for a growth experiment with juveniles of both snail forms without stressor substance and under natural feeding was conducted to evaluate its practicability and suitability. Besides growth, the endpoints survival and activity were analysed together with a post-experimental histopathological examination of the test animals. Juveniles of both forms of *T. fluviatilis* significantly grew over the 40-days experimental time, therefore we consider it practicable to analyse growth with the chosen design. No significant differences between the Northern-European and the Danubian form of *T. fluviatilis* were detected in the analysed endpoints growth, survival, activity, and histopathology, for what we conclude that the comparability of the two snail forms is given under laboratory conditions without stressor substance. Hence, our study provides a first step and a basis to analyse a possible intraspecific variability in *T. fluviatilis* considering its sensitivity towards environmental pollutants.

**Keywords** Gastropod, Intraspecific variability, Haplotype, Indicator organism, Pollutant sensitivity, Risk assessment

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## Introduction

Aquatic environments face a progressive contamination through the increase of the global human population accompanied by an intensification of biocide use. Atmospheric deposition, runoff, or soil leaching leads to an input of pollutants into freshwater habitats and to the exposure of aquatic organisms together with the accumulation of contaminants and their transfer along food chains (e.g. Fent 2013, Van Ginneken et al. 2017). Examining the consequences of environmental pollution through toxic chemicals poses a major challenge, and their potential impact has to be assessed properly and with reliable methods (Roubeau Dumont et al. 2019). In ecological risk assessment, traditional approaches analyse the effects of chemicals on ecologically relevant and most sensitive endpoints, i.e. mortality, reproduction, and growth, on a limited number of test species, e.g. in OECD protocols or the species sensitivity distribution approach (reviewed by Del Signore et al. 2016). Standard test species are often ubiquitous, widely geographically distributed, and generalists (Festing & Altman 2002). Usually, only one clonal strain per species and experiment is used, which assumes that one strain is representative for the entire species and rarely considers an intraspecific variability in the sensitivity towards contaminant exposure (Festing & Altman 2002, Petitjean et al. 2021). As the sensitivity towards toxicants can vary within species, as already shown e.g. for *Daphnia* (Barata et al. 2002), fish (Petitjean et al. 2021), and aquatic macrophytes (Roubeau Dumont et al. 2019), intraspecific variability is considered to be a potential source of error in standard toxicity testing (e.g. Côte et al. 2015). Therefore, several studies highlight the importance of considering intraspecific variability in ecological risk assessment to improve our understanding of the impact of chemicals on aquatic environments (Côte et al. 2015, Petitjean et al. 2021, Roubeau Dumont et al. 2019).

Data on intraspecific variation is rare, especially for gastropods (Salice & Roesijadi 2002), which are widely used indicator organisms for ecotoxicological testing (reviewed by Dhiman & Pant 2021). Due to their abundance in many types of freshwater environments, their various physiological reaction mechanisms towards toxicant exposure, and their limited mobility, snails are ecologically highly relevant organisms and inhabit a great biomonitoring potential (Mahmoud & Abu Taleb 2013, Otludil & Ayaz 2020). Environmental pollutants affect snail reproduction, growth, and mortality, which can be examined using biomarkers like food intake, growth rate, or behavioural changes (Dhiman & Pant 2021). Here, we use the freshwater gastropod *Theodoxus fluviatilis* (Linné, 1758) as test organism. The nerite snail is very common and native to all large rivers in Central Europe (Bunje 2005, Zettler 2008). It colonises running waters or the littoral zone of bigger lakes and prefers habitats with high oxygen and calcium contents and moderate salinity (Kangas & Skoog 1978, Zettler et al. 2004). The suitability of *T. fluviatilis* as test species has been mentioned in previous studies because it is easy to collect and maintain under laboratory cultures, and plays a key role in the functioning of freshwater ecosystems (Correia et al. 2013, Rothmeier et al. 2020). Being an almost exclusive biofilm grazer (Neumann 1961), it is potentially exposed to environmental pollutants via the dietary uptake of contaminated biofilms in addition to the respiratory uptake from ambient water. In inland freshwater habitats of Germany, two phylogenetically different forms of the species occur. The Northern-European form has been native to all large rivers, but shows a decline in abundance in the last decades and is nowadays only found in smaller tributaries of the River Rhine system in Southwestern and Central Germany (Zettler 2008). The Danubian form originates from populations in the Danube and Black Sea drainages and has probably been introduced to the River Rhine by

shipping through the Main-Danube canal (Gergs et al. 2015). The two forms of *T. fluviatilis* vary genetically in a haplotype of the mitochondrial cytochrome c oxidase subunit I (COI) gene (Bunje 2005, Gergs et al. 2015), and morphologically in size and shell patterns. A possible intraspecific variability between these two forms of the species considering differences in sensitivity towards environmental toxicants has not yet been studied.

Early life stages of animals are highly sensitive towards pollutants (Fent 2013). Furthermore, juvenile test individuals in ecotoxicological experiments enable to analyse the impact of toxicant exposure on growth, which is a highly ecologically relevant endpoint in toxicity testing (Bighiu et al. 2017, Del Signore et al. 2016). The impairment of growth is a response of organisms showing a general, stress-induced reaction due to suboptimal conditions and chronic stressors (Fent 2013), which gives the possibility of describing distinct exposure effects on population responses (Del Signore et al. 2016). To test if a comparative growth experiment can be conducted with juveniles of the two phylogenetically different forms of *T. fluviatilis*, a preliminary test design without stressor substance was conducted and evaluated in our study. We aimed at examining i) if juveniles of *T. fluviatilis* significantly grow under standardised laboratory conditions and natural feeding during an experimental time of 40 days, and ii) if there are significant intraspecific differences between the two forms of *T. fluviatilis* considering the endpoints survival, growth, activity, and histopathology.

## Methods and Materials

### *Test animals and maintenance*

We used two phylogenetically different forms of the snail species *T. fluviatilis*: the Northern-European and the Danubian form. Northern-European specimens were obtained from tanks at the German Environment Agency, which hatched from specimens originally sampled at the river Barolder Fließ (Germany). Danubian specimens were sampled from the German Upper River Rhine (kilometre 360). *T. fluviatilis* is an invertebrate species and not specially protected by the Federal Nature Conservation Act of Germany, thus no ethical permit was required for conducting our study. Individuals of the same form were kept together for 10 days of acclimatisation in tanks (volume 20 L) with water and naturally grown biofilm of a nutrient-poor gravel pit lake (Neureut, Germany). Air temperature and light intensity were held constantly (22 ± 1°C and 126 ± 35 lux, respectively), with a diurnal 12 h : 12 h day : night rhythm.

### *Experimental design*

Juvenile snails of 4.32 ± 0.53 mm shell length were used for the experiment, as a maximum shell length of 5 mm is considered for juvenile snails (B. Watermann, pers. comm.). Specimens of the two different forms were kept in threes in beakers with 400 mL filtered (30 µm) water (pH 8.19 ± 0.19) from the gravel pit lake. For both snail forms, beakers were replicated 8 times. Sexes are separate in *T. fluviatilis* with a sex ratio of 1:1 (Neumann 1959), thus it was likely that a balanced number of males and females were used for both forms. The duration of the experiment was 40 days from August to September 2020. As *T. fluviatilis* is a nearly exclusive biofilm grazer and has to shred diatoms by scraping them from a rough substrate with its radula before digestion (Neumann 1961), pebbles with naturally grown biofilm from the gravel pit lake were added as food source every 5 days. To avoid accumulation of feces and biofilm in the beakers, snails were transposed into clean beakers with new gravel pit lake water and pebbles every 10 days. Survival was checked daily by visual control. Snails not sticking at the pebble or beaker surface were stimulated with a needle and considered dead when showing no avoidance reaction, i.e. retraction into snail

shell. Dead individuals were removed from the beakers. To analyse snail growth, the shell length of every individual (Zettler et al. 2004, Fig. 1) was measured, which corresponds to the morphological length of the animal (Kirkegaard 2006). Shell lengths were measured at the beginning of the experiment and every media change date, i.e. every 10 days, with a digital calliper (L826.1, Roth, Germany) to the nearest 0.01 mm. Snail activity was examined visually every 8 experimental days. Each beaker was watched for 2 minutes noting how many individuals showed creeping activity.

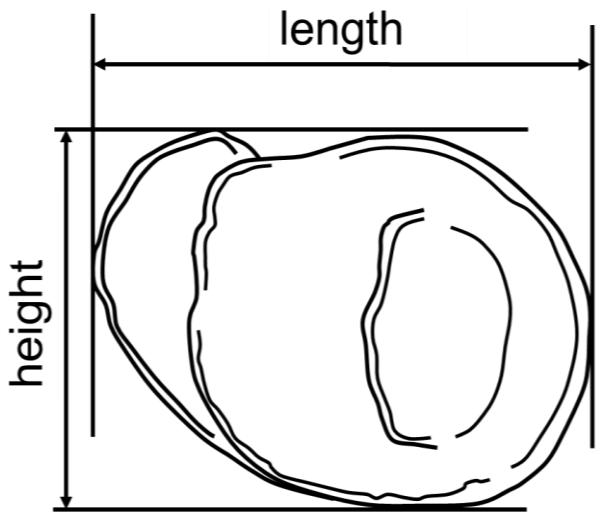


**Figure 1** Scheme of the shell underside of *Theodoxus fluviatilis* showing measurement distances of shell length and height. Modified after Zettler et al. (2004).

After 40 days, surviving individuals were transferred into bottles with gravel pit lake water for subsequent histopathological analysis. After three days of defaecation, they were narcotised in magnesium chloride (3%) before fixation in Bouin's fluid for 16 days, washed and stored in 80% ethanol (p.a.) and finally embedded in paraffin. Animal sections of 2–3 µm thickness were cut with a rotation microtome (Microm, Walldorf, Germany), put on glass slides and counterstained with haematoxylin and eosin (e.g. Mulisch & Welsch 2010, for detailed methodology see Rothmeier et al. 2020, Watermann et al. 2008). Histopathological examined structures were the gastrointestinal tract (including oesophagus, stomach, intestine, and digestive gland), as well as the kidneys and the cardiovascular system. Organic alterations which were considered pathologic were tissue dilatations and black or brown pigment depositions.

*Statistical analyses*

All calculations and statistical analyses were conducted with the program R (version 4.1.2, R Core Team 2021). The survival of the snails was examined using the Cox proportional hazards model (Fox & Weisberg 2011) of the package *survival* (version 3.2-10, Therneau 2021), which analyses and compares the risk to die for the two forms of *T. fluviatilis* during the experiment. Generalised linear models (GLM, error distribution = Gaussian) were conducted for the parameters growth and activity to analyse intraspecific differences between the two forms with experimental time as covariate. Snail growth was analysed as an increase of shell length during the experiment. Daily growth rate was calculated according to Gergs & Rothhaupt (2008) [shell length $_{(day\ n)}$ – shell length $_{(day\ n-10)}$/10] for surviving individuals of both snail forms. To analyse and compare the snail activity, the proportion of active individuals per form was calculated. For histopathological analysis, the proportion of surviving snails affected by pathological findings in tissues and organs after the experiment was calculated and Mann-Whitney-U-tests for independent samples were used to analyse the differences between the snail forms. Proportional data (activity and histopathology) was arcsine and square root transformed to homogenise variances.

## Results

In the group of the Northern-European form of *T. fluviatilis*, 79% of snails survived the experimental time, and in the group of the Danubian form of *T. fluviatilis*, 67% of snails survived (Fig. 2). The risk to die over the 40-day experimental time did not significantly differ between the two snail forms (Cox-regression: p = 0.29).

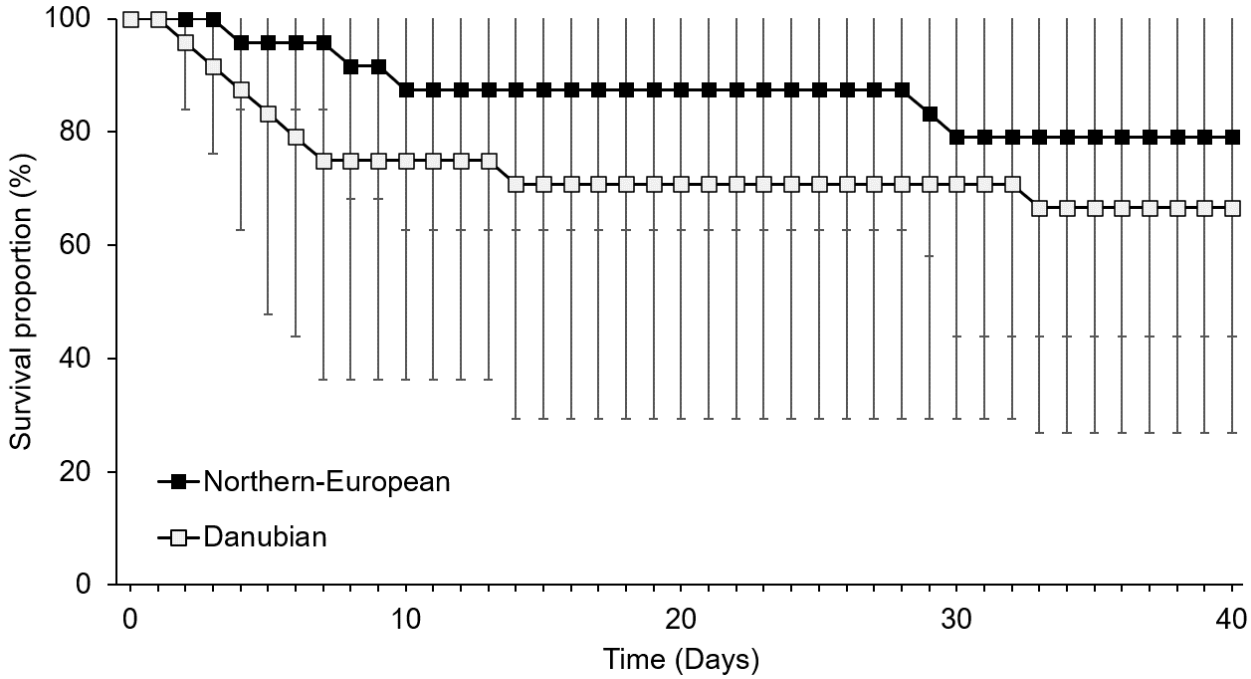


**Figure 2** Survival of juveniles of the freshwater snail *Theodoxus fluviatilis* (Northern-European and Danubian form, respective n = 8) in a 40-day laboratory experiment (survival recorded daily).

The shell length of both forms of *T. fluviatilis* increased significantly during the experiment (GLM: for Northern-European form p = 0.04 and for Danubian form p = 0.02), with no significant difference between the forms (GLM: p = 0.66; Fig. 3). In total, after the 40-days experimental time, the shell lengths of Northern-European snails had increased 0.57 (± 0.27) mm or 14 (± 7)%, and of Danubian snails 0.87 (± 0.70) mm or 21 (± 17)%. The mean shell growth rate (mm day$^{-1}$) did not significantly differ between the two forms of *T. fluviatilis* (GLM: p = 0.46; Fig. 4). The growth rate of both forms significantly increased over experimental time (GLM: for the Northern-European form p < 0.001 and for the Danubian form p = 0.01).

Snail activity did not significantly differ between the two forms of *T. fluviatilis* and over experimental time (GLM: p = 0.68). The histopathological analysis showed no pathologic alterations in intestines, kidneys, and the cardiovascular system of both forms. Black or brown pigment depositions in oesophagi and stomachs were found in 76 (± 25)% of the Northern-European and in 29 (± 30)% of the Danubian snails, which did not significantly differ between the two forms (Mann-Whitney-U-test: p = 0.23). Midgut gland dilatations were recorded in 10 (± 16)% of the Northern-European and in 29 (± 23)% of the Danubian snails, with no significant difference between the two forms (Mann-Whitney-U-test: p = 0.84).

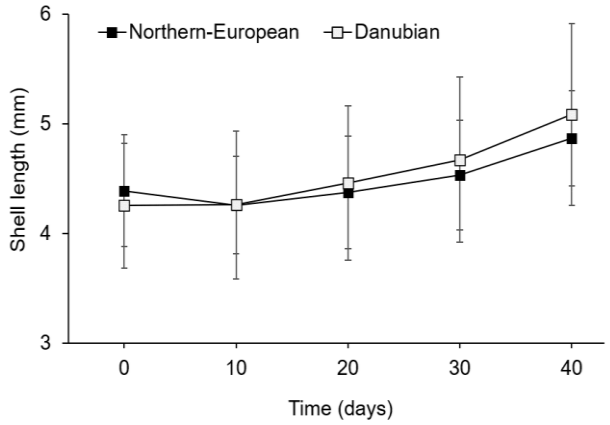


**Figure 3** Shell length (mm ± SD) of juveniles of the Northern-European and Danubian form of the freshwater snail *Theodoxus fluviatilis* (respective n = 8) fed on naturally grown biofilm over a 40-day laboratory experiment. Shell length was recorded in 10-days intervals.

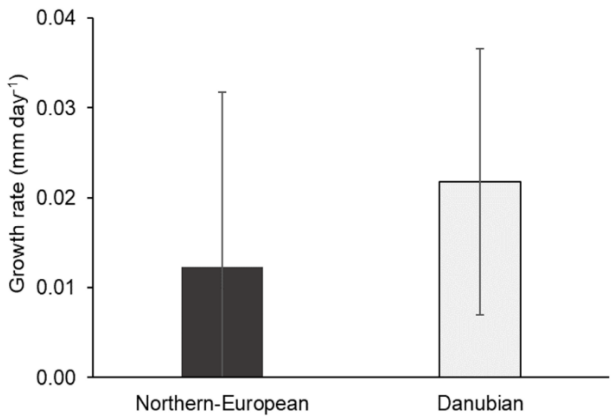


**Figure 4** Mean (± SD) shell growth rate (mm day$^{-1}$) of juveniles of the Northern-European and Danubian form of the freshwater snail *Theodoxus fluviatilis* (respective n = 8) fed on naturally grown biofilm in a 40-day laboratory experiment.

## Discussion

According to the results of our study, the proposed test design is suitable for a comparative growth experiment with juveniles of the Northern-European and the Danubian form of *T. fluviatilis*. As the test animals significantly grew during our experiment, we conclude that an experimental time of 40 days with natural feeding is sufficient to analyse the growth of juvenile snails of the two phylogenetically different forms. Besides growth, the endpoints survival and activity are practicable to be recorded and analysed with manageable effort in the experiment. We consider a histopathological analysis to be a meaningful and suitable tool to examine the physiology of test animals, as already suggested by Otludil & Ayaz (2020). The histological findings of pigment depositions in the oesophagi and stomachs and the midgut gland dilatations in the test animals are considered pathologic, nevertheless no impairments of survival, growth, or activity of the snails could be examined as possible consequences of physiological disturbances.

No significant differences between Northern-European and Danubian *T. fluviatilis* specimens considering survival, growth, activity, and histopathology were observed. Given the absence of intraspecific differences in our experiment without stressor substance, the comparability of the two forms under standardised laboratory conditions can be presumed. This provides the basis for a next step of testing a possible intraspecific variability in *T. fluviatilis* considering its sensitivity towards environmental toxicants. For gastropods, an intraspecific variation of responses towards pollutants has so far been reported e.g. for two strains of the freshwater snail *Biomphalaria glabrata* showing differences in cadmium sensitivity (Salice & Roesijadi 2002). Furthermore, Côte et al. (2015) found wide variation in copper tolerance within and between populations of the aquatic gastropod *Lymnaea stagnalis*, an important model organism used in ecological risk assessment. For *T. fluviatilis*, differences in salinity tolerance between brackish and freshwater populations have been reported (Kangas & Skoog 1978), which indicates that intraspecific variability exists in this species and could be assumed as well for a comparison of Northern-European and Danubian populations considering the sensitivity towards environmental toxicants.

Though intraspecific variation of organisms is an unquestionable biological reality, it is often

considered irrelevant or even neglected in ecological risk assessment (Côte et al. 2015). The Northern-European form of *T. fluviatilis* has always been an important indicator species for river monitoring in Germany, but is nowadays extinct in the River Rhine and has been replaced by the newly introduced Danubian form. To improve the ability to predict the effects of contaminants in aquatic environments (Petitjean et al. 2021), a possible intraspecific variation between the two forms should be considered in river assessment. Our study provides a first step towards an experimental design for a further investigation of intraspecific differences between the Northern-European and the Danubian form of *T. fluviatilis* considering its sensitivity towards environmental toxicants.

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