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Sensitivity and Stress of Groundwater Invertebrates to Toxic Pollution and Changes in Temperature

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Zusammenfassung

Das Grundwasser ist ein weitestgehend unerforschtes Ökosystem, das eine enorme Vielfalt an einzigartigen Organismen beherbergt. Darüber hinaus liefern Grundwasserökosysteme eine lebensnotwendige Grundlage für die Menschheit, indem sie große Mengen sauberen Wassers als Ressource für die Trinkwassergewinnung, für die Landwirtschaft und zur Aufrechterhaltung von industriellen Prozessen bereitstellen. Die ständig wachsende menschliche Bevölkerung geht mit immer größeren Nutzungsansprüchen einher, was zur Folge hat, dass mehrere ernstzunehmende Stressoren auf die Grundwasserökosysteme einwirken – übermäßige Grundwasserentnahme, Nährstoffbelastungen, toxische Kontaminationen, sowie Veränderungen des natürlichen Temperaturhaushalts. Diese Stressoren wirken sich auf die Organismen im Grundwasser aus und können damit auch potenziell das natürliche Funktionieren des gesamten Ökosystems bedrohen. Dies wiederum kann die Bereitstellung der vielfältigen Ökosystemleistungen (inklusive sauberer Wasser-Ressourcen) gefährden, die den Menschen täglich ohne Gegenleistung durch die Grundwasserökosysteme zur Verfügung gestellt werden.

Der Schwerpunkt dieser Dissertation lag auf der quantitativen Erfassung der negativen Auswirkungen von toxischen Schadstoffen und erhöhten Grundwassertemperaturen auf ausgewählte Grundwasser-Invertebraten. Zu diesem Zweck wurden zwei neue Verfahren für die Untersuchung von Schadstoff- und Wärmestress bei Stygofauna entwickelt – (i) ein ökotoxikologischer Test, mit dem die letalen Effekte von flüchtigen organischen Verbindungen untersucht werden können, sowie (ii) eine Methode für die Analyse von Ganzkörper-Katecholamin-Konzentrationen in Grundwasser-Organismen, anhand welcher physiologische Belastungen auf subletalem Niveau angezeigt werden können. Darüber hinaus wurden die Temperaturen ermittelt, welche kritisch für das Überleben zweier Krebstier-Arten sind, sowie die Frage untersucht, ob diese Organismen die unterschiedlichen Temperaturen in einem Wärmegradienten wahrnehmen können und folglich dazu in der Lage sind, ihren Aufenthaltsort entsprechend ihrer

Temperaturpräferenz bewusst auszuwählen, um Bereichen mit ungünstigen Temperaturverhältnissen fernzubleiben.

Das neu entwickelte ökotoxikologische Testverfahren wurde exemplarisch angewendet, um die Toxizität von Toluol für den Grundwasser-Flohkrebs *Niphargus inopinatus* zu untersuchen. Die Ergebnisse zeigten, dass die sogenannte „ultimate" LC_{50}, d.h. diejenige Toluol-Konzentration, die per Definition für einen „durchschnittlichen" *Niphargus* auf Dauer tödlich wäre, durchaus im Bereich der Toluol-Konzentrationen liegt, die häufig an kontaminierten Standorten vorgefunden werden. Gleichermaßen war die in Deutschland aktuell empfohlene maximale Einleitungstemperatur beim Betrieb von oberflächennahen Geothermie-Anlagen (20°C) auf Dauer kritisch für das Überleben der untersuchten Grundwasser-Asseln und –Flohkrebse. Demzufolge können die Stressoren, denen Grundwasserfauna in der heutigen Zeit ausgesetzt ist, durchaus von relevantem Ausmaß sein und eine ernstzunehmende Gefahr für das Überleben dieser Organismen darstellen.

Die unterschiedlichen Arten, die in dieser Dissertation untersucht wurden, waren unterschiedlich empfindlich gegenüber Temperaturstress. Es stellte sich heraus, dass die Grundwasserassel *Proasellus cavaticus* empfindlicher gegenüber einer Erhöhung der Grundwassertemperatur als der Flohkrebs *Niphargus inopinatus* ist und somit erwiesen sich bei den Asseln bereits niedrigere Temperaturen als tödlich. Darüber hinaus waren beide Arten in der Lage, die unterschiedlichen Temperaturen in einem Wärmegradienten wahrzunehmen und wählten einen geeigneten Aufenthaltsort entsprechend ihrer Temperaturpräferenzen.

Um die Frage beantworten zu können, ob kurzzeitiger, subletaler Temperatur-stress anhand einer Veränderung im Katecholamin-Gehalt aquatischer Flohkrebse festgestellt werden kann, wurde zunächst das Vorkommen dieser Substanzen bei Oberflächen- und Grundwasser-Flohkrebsen untersucht. In einem nächsten Schritt wurden die Veränderungen der Katecholamin-Konzentrationen als Folge von Wärmestress analysiert. Das Vorhandensein von Dopamin, Noradrenalin und Adrenalin im stygobionten Flohkrebs *Niphargus inopinatus* konnte zum ersten Mal erfolgreich mittels zweier unabhängiger Methoden nachgewiesen werden. Die gefundenen Ganzkörper-Katecholamin-Konzentrationen waren erstaunlich hoch im Vergleich zu den Konzentrationen, die im verwandten Oberflächenwasser-

Flohkrebs *Gammarus pulex* gemessen wurden. Bei beiden untersuchten Arten veränderten sich die Katecholamin-Gehalte nach der Einwirkung von kurzzeitigen, subletalen Temperatur-Erhöhungen und zeigten somit an, dass die Katecholamine an der physiologischen Stress-Antwort dieser Organismen beteiligt sind. Es waren jedoch Unterschiede im Muster der Katecholamin-Konzentrationen beider Flohkrebs-Arten zu beobachten. Bei *G. pulex*, führte der Wärmestress zu einer Erhöhung der Noradrenalin-Konzentrationen. Im Unterschied dazu, trat bei *N. inopinatus* eine Erhöhung der Adrenalin-Werte auf, was darauf hindeutet, dass es bei der Grundwasser-Art zu einer schnelleren Umwandlung der Katecholamine ineinander gekommen sein könnte.

Aufgrund der charakteristischen Anpassungen von Grundwasserfauna an ihren Lebensraum, besitzen diese Organismen eine besonders hohe Vulnerabilität gegenüber Einwirkungen von anthropogenen Stressoren. Darüber hinaus führt der hohe Anteil endemischer Arten, die über ein sehr kleinräumliches Verbreitungsareal verfügen, zu einem hohen Extinktionsrisiko, was darin begründet liegt, dass das lokale Aussterben von Populationen schnell zu einem absoluten (d.h. weltweiten) Aussterben einer Art führen kann.

Die Stressoren, die in dieser Dissertation untersucht wurden, können *in situ* in so hohen Größenordnungen auftreten, dass eine ernste Gefährdung für das Überleben von Grundwasser-Invertebraten entsteht. Des Weiteren haben die Ergebnisse dieser Arbeit gezeigt, dass unterschiedliche Arten unterschiedlich empfindlich gegenüber Stressoren reagieren können, und zwar trotz der relativ stabilen Umweltbedingungen, die natürlicherweise im Grundwasser vorherrschen. Um die Entwicklung von nachhaltigen, ökologisch-begründeten Grundwassernutzungs- und Bewirtschaftungsstrategien voranzutreiben, sowie für die Erarbeitung geeigneter Schutzmaßnahmen und –Pläne, sollten weitere Studien durchgeführt werden, in denen die Empfindlichkeit von Stygofauna gegenüber anthropogen verursachten Stressoren, sowie der Einfluss solcher Stressoren auf Populations- und Lebensgemeinschafts-Niveau erforscht wird. Der unschätzbare Wert der Grundwasserökosysteme, ihrer Lebewesen, sowie der Ökosystemleistungen, die sie der menschlichen Gesellschaft zur Verfügung stellen, erfordern die Implementierung von angemessenen, gesetzlich geregelten und umfassenden Maßnahmen zum Schutz ihres Fortbestehens.

Summary

Groundwater is a vastly unexplored realm, offering habitat to a great diversity of unique species. Moreover, groundwater ecosystems are of vital importance for the sustaining of human life as they offer immense supplies of clean water used for drinking, irrigation, as well as for the support of industry. The constantly growing human population poses increasing pressures to groundwater utilization and results in several serious anthropogenic stressors that act on groundwater ecosystems – groundwater (over-)abstraction, nutrient loading, toxic pollution, as well as changes in the aquifers' natural temperature regime. These stressors affect the organisms living in groundwater and with this, potentially also the natural functioning of the entire ecosystem. In turn, this can threaten the provision of the various ecosystem services and goods supplied to humans for free by groundwater ecosystems (including also clean water resources).

The focus of the present dissertation thesis was laid on the quantification of the negative effects that are caused by toxic compounds and elevated temperatures to selected groundwater species. To this end, two novel tools for the assessment of toxic and temperature-related stress on stygofauna were developed – a bioassay for testing the toxicity effects of volatile organic compounds on the survival of stygobites, and a method for the quantification of whole-animal catecholamine concentrations indicating physiological stress at the sublethal level. Moreover, the temperatures that are critical for the survival of two species of groundwater crustaceans were assessed and it was tested whether these species are able to perceive different temperatures in a heat gradient and choose a preferred residence location in order to escape from unfavourable conditions.

An exemplary assessment of the toxicity of toluene towards the stygobitic amphipod *Niphargus inopinatus* revealed that the ultimate LC_{50} for this organism, *i.e.* the concentration which would kill the average niphargid on long exposure, lies well within the range of toluene concentrations that frequently occur *in situ*, at contaminated field sites. Similarly, the temperatures that were lethal for 50% of the tested stygobitic amphipods and isopods on the long term were within the range

of temperatures that are currently recommended for the operation of shallow geothermal installations in Germany. Therefore, the stressors that are nowadays encountered by groundwater fauna can be of a magnitude which is within the critical range for survival and can therefore pose a serious threat to these organisms.

Different species had different sensitivities towards temperature stress. The stygobitic isopod *Proasellus cavaticus* was more susceptible towards temperature elevation than the amphipod *N. inopinatus* and thus, experienced mortality at lower temperatures. Furthermore, both species were able to sense areas with different temperatures and choose a residence spot according to their specific temperature preferences.

In order to investigate whether short-term temperature stress at the sublethal level can be assessed via a change in the catecholamine levels of aquatic amphipods, the presence of catecholamines in surface water and groundwater amphipods was investigated. As a next step, the changes in the amphipods' catecholamine levels in response to heat stress were measured. The presence of the biogenic amines dopamine, noradrenaline and adrenaline in the stygobitic amphipod *N. inopinatus* was successfully demonstrated for the first time via two independent methods. The whole-tissue catecholamine concentrations were surprisingly high as compared to the amounts found in *Gammarus pulex*, a related surface water amphipod. In both species, the catecholamine levels changed in response to sudden, short-term temperature elevations, thus demonstrating that catecholamines are involved in the physiological stress response of these organisms. However, the observed patterns differed between the two species. In *G. pulex*, the heat stress resulted in an increase in noradrenaline levels. In contrast, in *N. inopinatus* an increase in average adrenaline levels occurred, indicating that the sequential catecholamine conversion steps might take place faster in the stygobitic amphipod.

Due to the characteristic adaptations of obligate groundwater fauna to their habitat, these organisms are particularly vulnerable towards the effects of anthropogenic stressors. What is more, the exceptionally high percentage of short range endemic species in groundwater poses high risks that local extinctions of populations may become equivalent to the full extinction of a species worldwide.

The stressors investigated in this dissertation thesis occur *in situ* at magnitudes that pose realistic threats to the survival of groundwater invertebrates. Furthermore, the results of the thesis showed that despite the relatively stable environmental conditions that naturally occur in groundwater, different species have different susceptibilities towards stress. Therefore, in order to support the development of ecologically sound groundwater management and conservation strategies, further research on the range of the species' stress-sensitivities to anthropogenic stressors, as well as on the impacts of stressors on the population and community levels should be performed. The high value of groundwater ecosystems, of their species, as well as of the ecosystem services they provide for human society, call for appropriate measures for their legal protection.

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List of Publications and Contributions

Publications:

I. **Avramov, M.**, Schmidt, S. I., Griebler, C. (2013). A new bioassay for the ecotoxicological testing of VOCs on groundwater invertebrates and the effects of toluene on *Niphargus inopinatus*. *Aquatic Toxicology*, 130-131, pp. 1-8.

II. Brielmann, H., Lueders, T., Schreglmann, K., Ferraro, F., **Avramov, M.**, Hammerl, V., Blum, P., Bayer, P., Griebler, C. (2011). Oberflächennahe Geothermie und ihre potenziellen Auswirkungen auf Grundwasserökosysteme. *Grundwasser*. 16, 77-91.

III. Pfister, G., Rieb, J., **Avramov, M.**, Rock, T. M., Griebler, C., Schramm, K.-W. (2013). Detection of catecholamines in single individuals of groundwater amphipods. *Analytical and Bioanalytical Chemistry*, 405, 5571-5582.

IV. **Avramov, M.**, Rock, T. M., Pfister, G., Schramm, K.-W., Schmidt, S. I., Griebler, C. (2013). Catecholamine levels in groundwater and stream amphipods and their response to temperature stress. Accepted for publication in *General and Comparative Endocrinology*.

The **fifth publication** that arose during the course of this dissertation project is only listed and cited here, but not embedded in full length within the text of the thesis:

V. **Avramov, M.**, Schmidt, S. I., Griebler, C., Hahn, H. J. & Berkhoff, S. (2010). Dienstleistungen der Grundwasserökosysteme. *Korrespondenz Wasserwirtschaft*. 2, 74-81.

My contribution[1] to the publications included in the thesis:

I. All experiments involved in the development, optimization and exemplary application of the bioassay were designed and conducted by me, under the supervision and advice of Dr. C. Griebler and Dr. S. I. Schmidt. The manuscript was written by me and revised by Dr. S. I. Schmidt and Dr. C. Griebler.

[1] All publications presented here resulted from the joint efforts of all contributing authors. For the purposes of this dissertation, mainly those contributions are pointed out, in which I was involved. The full contributions of the other authors are not given in detail.

II. I was involved in the conceptual design of the temperature gradient experiment performed by K. Schreglmann, as well as the temperature dose-response studies performed by K. Schreglmann and F. Ferraro. The diploma thesis of K. Schreglmann (2010) and the master thesis of F. Ferraro (2009) were co-supervised by me. Hence, I gave methodological guidance, contributed to the data interpretation and was furthermore involved in the statistical analysis of the data as well as the taxonomic species determination.

III. I was involved in the conceptual development of the project (under the advice of Dr. C. Griebler and Dr. S. I. Schmidt), performed the sampling, determined the amphipod species, and was strongly involved in the data interpretation. Dr. G. Pfister, J. Rieb, T.M. Rock, and Prof. Dr. K.-W. Schramm developed the analytical methodology, conducted the catecholamine analysis, and prepared a first sketch of the manuscript. I contributed several sections to the manuscript and was substantially involved in the further development, writing and editing of the manuscript.

IV. The study concept was mainly developed by me, and I also designed the experimental setup, performed the experiment and interpreted the data (under the advice of Dr. C. Griebler and Dr. S. I. Schmidt, and taking into consideration input from the other co-authors). Dr. G. Pfister, T. M. Rock, and Prof. Dr. K.-W. Schramm conducted the catecholamine analysis including method optimization and quality assurance/ quality control. They also assisted in the development of the concept and contributed one part of the method section of the manuscript. The paper was written by me and revised by all co-authors.

1. Introduction

1.1. Groundwater biota

The subterranean realm below our feet harbours unique, yet vastly unexplored ecosystems with a great diversity of organisms. According to an estimation by Culver and Holsinger (1992), there are between 50,000 and 100,000 obligate subterranean karstic and cave-dwelling animal species worldwide, including both the aquatic and terrestrial organisms. This number does not account for the fauna from porous aquifers, so that in total the subterranean species are probably even more numerous. Precise figures are difficult to obtain, since new species (particularly crustaceans) are being continuously described at a high rate (Stoch & Galassi, 2010) and many regions of the world are still insufficiently explored. With respect to aquatic subterranean fauna (*i.e.* stygofauna), in the year 2002 there was still a lack of profound taxonomic knowledge in most taxonomic groups (Gibert & Deharveng, 2002). Even though major advancements have been achieved since then, *e.g.* within large-scale coordinated surveys such as the European PASCALIS[2] project, this still holds true today. Thus, even after PASCALIS, over 50% of the stygobitic[3] species in European biodiversity hotspots were estimated to have remained undiscovered (Deharveng *et al.*, 2009). Nevertheless, these recent biodiversity assessments have demonstrated that groundwater ecosystems are characterized by an exceptional richness in short-range endemic species and a high level of relict taxa (Humphreys, 2000; Deharveng *et al.*, 2009; Eberhard *et al.*, 2009). In addition, several orders of crustaceans (*e.g.* Bathynellacea and Thermosbaenacea) can be found exclusively in groundwater (Sket, 1999; Danielopol *et al.*, 2003).

In Europe and worldwide, the Crustacea are the most diverse stygobitic group, making up for more than 70% of global, and 65% of European groundwater species richness (Holsinger, 1993; Stoch & Galassi, 2010). In particular, amphipods, isopods and copepods are among the most abundant, widespread and

[2] PASCALIS: Protocols for the Assessment and Conservation of Aquatic Life In the Subsurface
[3] stygobitic: obligate groundwater fauna that complete their entire life cycle in subterranean habitats

taxonomically diverse orders (Gibert & Deharveng, 2002). Moreover, molluscs, water mites, nematodes, oligochaetes, flatworms, and many other invertebrates can also be found in groundwater ecosystems. In habitats offering an extended living space, *e.g.* fractured rock aquifers or caves in karstic systems, also bigger animals may occur, such as fishes and salamanders.

As a result of the permanent darkness in aquifers, no photosynthetic activity is possible, thus preventing the colonization by primary producers (*i.e.* algae and higher plants). Hence, groundwater food webs are dependent on the input of oxygen and particulate and dissolved organic matter percolating from the surface, and as such, the biocoenoses are mostly heterotrophic. Some exceptions can be found in chemoautotrophically sustained communities, for example in the Movile cave system, Romania – a highly productive ecosystem based on the carbon fixation by hydrogen sulphide-oxidizing microorganisms (Sarbu *et al.*, 1996). Apart from this, if anthropogenically unaffected, most of the underground habitats are oligotrophic and sparsely populated (Gibert *et al.*, 1994; Gibert & Deharveng, 2002).

Obligate groundwater organisms are specifically adapted to suit the living conditions in aquifers, comprising not only scarce, patchily distributed food and low concentrations of nutrients, but also darkness, temporarily occurring hypoxia, and (at least in temperate regions) relatively low but stable temperatures (Coineau, 2000). Accordingly, stygobites are characterized by a reduced metabolism, low growth and reproduction rates, lack of eyes and pigmentation, and the ability to withstand hypoxia and starvation to a higher extent than related surface water species (Hervant *et al.*, 1995; Schminke, 1997; Spicer, 1998; Hervant *et al.*, 1999; Simčič *et al.*, 2005).

The harsh living conditions in groundwater are also reflected by the characteristic structure of subterranean food webs. Due to the absence of primary producers and herbivores, groundwater food webs have been described as 'truncated' at the bottom (Gibert & Deharveng, 2002). Moreover, based on the scarcity and the irregular availability of food, it has been suggested that an evolutionary shift in the feeding strategy of predators towards omnivory has occurred, resulting in an almost complete absence of obligate predators in groundwater ecosystems (Gibert & Deharveng, 2002). Instead, a pronounced specialization on the ability to utilize

various types of food source, as well as on the resistance to starvation, is assumed to have taken place.

The resulting high proportion of detritivores/ omnivores is essential for the organic matter decomposition and nutrient cycling in groundwater ecosystems. Remineralized nutrients (as well as water) are delivered to the above-ground streams and groundwater-dependent ecosystems such as floodplains and wetlands via groundwater discharge, thus promoting the organisms that live on the surface (Hancock *et al.*, 2005). This 'support of groundwater dependent ecosystems' is one of the ecosystem services[4] provided by aquifers and their biota (Hayashi & Rosenberry, 2002; Tomlinson & Boulton, 2010). Other services and goods (see Fig. 1, page 5) include *inter alia* groundwater biodiversity itself, flood mitigation, drought attenuation, organic matter breakdown, as well as contaminant degradation, and consequently – the storage and provision of clean water resources (Danielopol *et al.*, 2003; Millennium Ecosystem Assessment, 2005; Boulton *et al.*, 2008; Avramov *et al.*, 2010). The importance of these ecosystem services for humankind is evident, one of the most prominent examples being the fact that an estimated 2 billion of people worldwide are dependent on groundwater for their drinking water supplies (Morris *et al.*, 2003). At the same time, our knowledge on the processes underlying the provision of groundwater ecosystem services and goods is still far from being complete. Particularly, the scientific understanding of how groundwater invertebrates are involved in these processes and to what extent species richness plays a role, is 'almost inexistent' (Gibert & Deharveng, 2002; Boulton *et al.*, 2003; Boulton *et al.*, 2008). For example, it is well established that groundwater microbial communities are the key performers in terms of pollutant biodegradation in contaminated aquifers (e.g. Haack & Bekins, 2000; Lovley, 2001; Röling & van Verseveld, 2002; and recently: Herzyk *et al.*, 2013). In addition, it has been shown that protozoa that are grazing on the degrader populations can have a strong influence on biodegradation by ultimately causing either a stimulation (Mattison *et al.*, 2005) or inhibition (Kota *et*

[4] As defined by G. Daily (1997), ecosystem services are 'the conditions and processes through which natural ecosystems, and the species that make them up, sustain and fulfil human life. They maintain biodiversity and the production of ecosystem goods, such as seafood, forage, timber, biomass fuels, natural fiber, and many pharmaceuticals, industrial products, and their precursors'. Reference: Daily, G. C. (1997). What are ecosystem services? In Nature's Services: Societal Dependence on Natural Ecosystems. Island Press, Washington, D.C.

al., 1999; Cunningham *et al.*, 2009) of biodegrader activities, as well as by reducing the bacterial clogging of the sediments (Mattison *et al.*, 2002). In comparison, the role of invertebrates is not so well characterized yet. It has been suggested however, that stygofauna may contribute to the maintenance of hydraulic conductivity (Husmann, 1978; Danielopol, 1989) and improve the substrate availability for microbes through their burrowing and bioturbation activities (Gibert & Deharveng, 2002; Mermillod-Blondin *et al.*, 2003; Nogaro *et al.*, 2006), breakdown of coarse particulate organic matter, excretion of nutrients, as well as pelletisation (Danielopol, 1989; Boulton *et al.*, 2008). This is expected to enhance the decomposition of natural organic matter and potentially also contaminant biodegradation – provided that the organisms involved are able to withstand the toxicity of the contaminants, which was true for the protozoan studies mentioned above. Moreover, various groundwater invertebrates *e.g.* nematodes and oligochaetes, but also copepods (Hancock *et al.*, 2005), amphipods and isopods (Sinton, 1984), are known to feed on bacteria, and have been thus (in analogy to protozoa) suggested to stimulate bacterial growth rates and potentially also support contaminant degradation (Ward *et al.*, 1998; Tomlinson & Boulton, 2010). Last, but not least, Sinton (1984) showed via gut content analyses that groundwater isopods and amphipods directly contributed to the pathogen removal in a sewage polluted aquifer through the ingestion of coliform bacteria. All these examples demonstrate a variety of mechanisms through which stygofauna may contribute to the functioning of groundwater ecosystems, and consequently, also to the provision of groundwater ecosystem services utilized by humankind. However, many of the studies mentioned above are based on evidence from settings without a pronounced carbon and nutrient limitation such as the hyporheic zone of rivers, shallow aquifers, or laboratory mesocosms. The question whether these mechanisms apply in a similar manner to deeper and more energy-limited aquifers, is a research gap that has been pointed out on several occasions (e.g. Gibert & Deharveng, 2002; Boulton *et al.*, 2003; Boulton *et al.*, 2008; Humphreys, 2009) and needs to be addressed in the future.

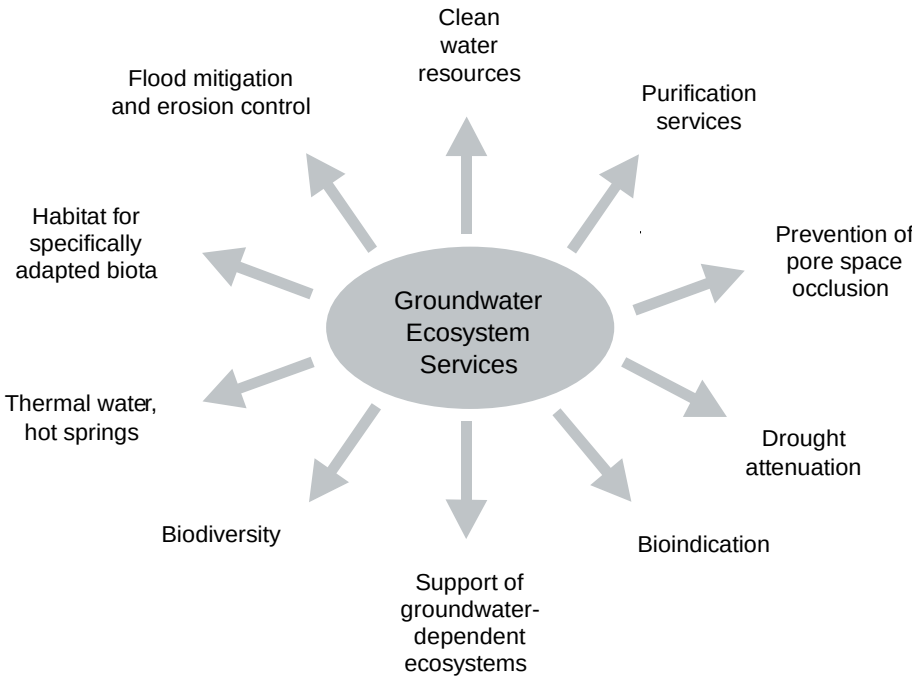

Figure 1: Services and goods provided by groundwater ecosystems, modified after Avramov *et al.* (2010).

1.2. Anthropogenic stressors acting on groundwater ecosystems

It is widely agreed that ecosystem services and goods (Fig. 1) can only be provided as long as the ecosystem's functions are not impaired (e.g. Herman *et al.*, 2001; Danielopol *et al.*, 2004). This implies that anthropogenic disturbances of the habitat and the living conditions of groundwater biota should not exceed a certain critical threshold defined by the specific resistance and resilience of the ecosystem. However, as has been pointed out by Masciopinto *et al.* (2006), 'the demographic growth in developing countries and the increasing pressure of anthropogenic activities in industrialized states around the world are leading to a gradual contamination of the natural habitats on our planet'. It is therefore becoming more and more important to investigate how stressors affect ecosystems and where the critical thresholds for these stressors are.

In scientific literature, as well as in general public perception, there are two different points of view regarding stressors – one focusing on the fact that groundwater is an invaluable resource sustaining human well-being, and the other one recognizing aquifers as precious ecosystems. This has important

implications for the categorization of stressors and for the way they are handled. For example, while pathogenic microorganisms or indicators for faecal contamination such as coliform bacteria may be regarded as stressors in terms of groundwater hygienic quality, they are not necessarily harmful to groundwater biota – on the contrary, they might even represent an additional food source for these animals (e.g. Sinton, 1984). Hence, while the stressors identified from a "resource-oriented" point of view may pose a threat to human health and lead to economic burdens associated with the need for water treatment activities, the "ecosystem-oriented" consideration reveals those kinds of stressors that have an ambivalent mode of action. On the one hand, they directly affect groundwater biota and biotic interactions, and on the other hand (on longer time scales and indirectly), impairment of ecosystem functions again leads to negative outcomes for humanity. While both views on stressors have their merits, in the context of this work the focus was laid on those stressors that affect the natural state and functioning of groundwater ecosystems, thus adopting the "ecosystem-oriented" view. These stressors can be grouped into four main categories: 1) nutrient loading; 2) groundwater abstraction/ overexploitation; 3) toxic pollution; 4) changes in natural temperature regime (see Fig. 2). The research included in this thesis focused on two of them, *i.e.* the stress caused to groundwater fauna as a result of (i) toxic pollution and (ii) changes in natural temperature regime (see section 2). Nevertheless, as all four groups of stressors may strongly influence groundwater ecosystems and since furthermore, the effects of these stressors can be interrelated, they will all be briefly introduced.

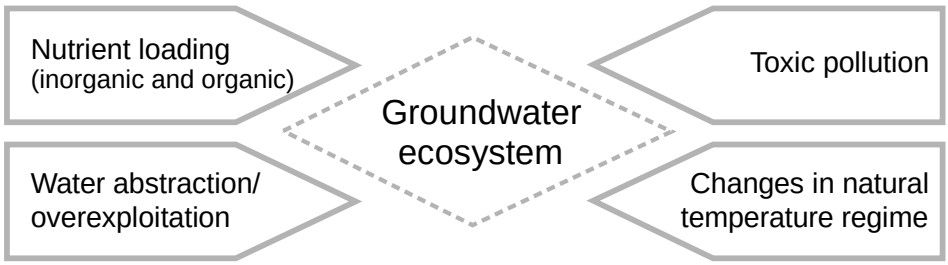

Figure 2: Anthropogenic stressors affecting groundwater biota and the functioning of groundwater ecosystems.

1.2.1. Nutrient loading

Nutrient loading (in the sense of this thesis) comprises all anthropogenically derived inputs of non-toxic organic and inorganic material that percolate into the groundwater (*e.g.* due to agricultural practice, artificial recharge, stormwater infiltration), and that can be used either by fauna or by the microbial communities for growth. This context is different from surface water bodies, where the term 'nutrients' mainly refers to inorganic nitrogen and phosphorous compounds that lead to eutrophication and enhanced primary production (*e.g.* algal blooms). In groundwater ecosystems nitrogen and phosphorous are not primarily an adverse issue: in darkness, photosynthesis is prevented, and under reducing conditions, nitrate can be used as an alternative electron acceptor by microbes when the amount of dissolved oxygen is too low for aerobic respiration. Thus, high nitrate concentrations in groundwater are mainly of concern for humans – in terms of drinking water production. However, regarding groundwater biota, nitrate can also have (indirect) implications. An increase in groundwater nitrate concentrations is considered to reflect a high hydrological connectivity to the surface (*e.g.* Schmidt *et al.*, 2007; Stein *et al.*, 2010), and hence, aquifers with anthropogenically increased nitrate levels often also have elevated loads of dissolved and particulate organic carbon that originate from above. A higher amount of dissolved organic carbon (DOC) in groundwater (as long as it is readily bioavailable) can lead to a stimulation of bacterial growth, which in turn can cause pore space occlusion, as well as the depletion of dissolved oxygen and eventually, the elimination of stygofauna. For example, it has been reported that in a well subjected to intermittent but heavy effluent pollution from a sewage irrigated area overlying a gravel aquifer, the entire macroinvertebrate population was killed (Sinton, 1984). In this well, over 300 dead and decaying crustaceans were found but no single live animal. The author assumed that this dramatic mortality had occurred as a result of rapid oxygen depletion in the heavily contaminated well water. Similarly, Wood *et al.* (2008) observed that after a pollution event with organically rich material (paper pulp, as well as peat from a water treatment plant) in a cave stream, no benthic invertebrates could be found anymore, while

previously a subterranean community of 34 stygophilic[5] and stygoxene[6] species had been present.

Such devastating effects are not the only possible outcome, when organic contamination acts as a stressor on groundwater ecosystems. As long as oxygen levels remain sufficiently high, an increased load of organic carbon can also lead to an increase in groundwater fauna density and diversity (*e.g.* as observed by Datry *et al.* (2005) and Sket (1977)). Boris Sket (1999) even expressed the view that slight organic pollution may be to some extent favourable for subterranean dwellers (in terms of providing them with additional energy sources). Nevertheless, even if faunal biomass production is stimulated, a change in community structure can alter biotic interactions and thus, affect ecosystem functioning. Furthermore, given sufficient hydrological connectivity, a higher productivity of the ecosystem due to increased availability of carbon is assumed to cause a displacement of stygobitic species by non-stygobites that are invading from the surface and are strong competitors for food (Sket, 1999). The reason for this is the low metabolism and slow reproduction of true stygobites. Being K-strategists, they are not able to quickly reproduce and establish high population numbers, thus failing to efficiently exploit a new energy source if it is available only for a short period of time. In support of this species displacement assumption, Stein *et al.* (2010) found a positive correlation between the abundance of non-stygobites in porous aquifers and those parameters that indicated a strong influence from agricultural land use on the surface (*i.e.* the concentration of nitrate, the bacterial abundance and the amount of particulate organic matter). However, while well-accepted by many authors, this view has been difficult to prove, as often organic pollution causes several factors to act simultaneously on groundwater fauna composition and hence, factors other than competition might be the more important ones. For example, Malard *et al.* (1996) pointed out that while the establishment of a dense population of epigean beetles in a cave occurred together with organically polluted infiltrating water (as described in an earlier publication: Malard *et al.*, 1994), it was not clear whether the stygobites of

[5] stygophilic: species that occur temporarily in subterranean aquatic environments and may complete certain parts of their life cycle there.

[6] stygoxene: species that usually live in surface water habitats and are only occasionally/ accidentally present in groundwater.

the cave were killed/ moved away as a result of the (putatively harmful) contaminants or because they were unable to successively compete with the epigean species. Without doubt, in order to better understand or even predict the impacts of nutrient loading on groundwater ecosystems, and also for the design and implementation of effective conservation strategies, further research on this topic is needed.

1.2.2. Groundwater abstraction/ overexploitation

Groundwater abstraction leads to aquifer depletion whenever the abstracted amounts exceed the amounts renewed. If this overexploitation lasts for a long time and affects extensive areas, persistent groundwater depletion can occur (Wada et al., 2010). Many aquifers are very slowly renewed and therefore, can effectively be regarded as 'non-renewable on human timescales' (Gleeson et al., 2010). At the same time, the overexploitation of such slowly replenished aquifers can have severe social, environmental and economic consequences (Gleeson et al., 2010). From a global perspective, this is particularly striking. A recent study estimated that 39% (*i.e.* 283 ± 40 km^3 out of 734 ± 82 km^3) of the global yearly groundwater abstraction in the year 2000 were overdraft (Wada et al., 2010). The same study also showed that compared to the 1960s, both the abstraction rate and the groundwater depletion have more than doubled and are likely to increase further in future. The consequences of this overexploitation for humanity are obvious and are of great concern to governments all over the world as reflected by the UN Millennium Declaration (UN, 2000). In this document, the international community declared its resolution to "stop the unsustainable exploitation of water resources by developing water management strategies at the regional, national and local levels, which promote both equitable access and adequate supplies".

Compared to the concerns on quantitative issues, the ecological consequences of groundwater abstraction on aquifers and groundwater-dependent ecosystems have received by far less attention. However, as stated by Wada *et al.* (2010), lowering the groundwater level may lead to land subsidence and salt water intrusion in deltaic areas, and furthermore have devastating effects on natural streamflow, groundwater-fed wetlands and related ecosystems. Supporting this, Benejam *et al.* (2010) detected clear effects of altered flow regimes on stream-fish

assemblages in Mediterranean streams that were profoundly altered by water abstraction either directly or via groundwater withdrawal. The effects observed included reduced population densities, fewer benthic species, and reduced occurrence and abundance of intolerant species. Similarly, in the Gnangara Groundwater System in Western Australia, an area with previously rich terrestrial mammalian fauna, but heavily impacted by declining rainfall and increased aquifer abstraction during the last 30 years, only 11 out of the 28 historically recorded terrestrial native mammals were found in the year 2012 (Wilson et al., 2012). Regarding subterranean ecosystems and stygofauna, even less information is available on the effects of groundwater abstraction and changes in flow rates (Humphreys, 2009). However, it is clear that in aquifers too, groundwater depletion can lead to the loss of aquatic habitat, and in turn to losses of populations, species, and ecosystem processes and services (as reviewed by Larned, 2012). On a local scale, intense groundwater pumping can lead to relatively rapid shifts of groundwater level, as for example reported from the Baget karst system (Ariège) in Southern France. Here, the water level was lowered by as much as 21 m below the original water table (within 4 days during a series of high discharge pumping tests), leading to an increased drift of the microcrustacean fauna (mainly harpacticoid copepods). As a consequence, the pumping site was partially depopulated and the harpacticoid population of the drainage in the down-stream part of the system was also disturbed (Rouch *et al.*, 1993). A recent study by Stumpp and Hose (2013, submitted) showed that even less pronounced water table drawdowns that are similar to the natural decline rates, can already lead to the stranding of stygofauna in the newly formed unsaturated zones. In column experiments, these authors observed that up to 19% of the tested Syncarida and even 88% of the cyclopoid copepods remained stranded as a result of a water table decline of 2.6 m d^{-1}. Additionally, the study demonstrated that once stranded, the animals are very likely to die due to desiccation. The ultimate effects of water abstraction on stygofauna *in situ* will depend on several factors, *e.g.* (i) whether drawdown proceeds beyond the zones suitable as a living space (Humphreys, 2009), (ii) whether the animals can move quickly enough in order to follow the water, or (iii) whether they get caught in pores that become disconnected from the main flow paths (Stumpp & Hose, 2013,

submitted). However, keeping in mind that anthropogenic groundwater abstraction can lead to much larger, permanent water table declines of more than 100 m in just 20 years (as depicted in Custodio (2002) for different regions in Spain), the severe impacts that overexploitation can have on groundwater ecosystems are evident.

1.2.3. Toxic pollution

Major sources of ***toxic pollution*** in aquifers include leaching of agricultural chemicals from cultivated areas as well as leakages from underground storage tanks for liquid chemicals and fuels, chemical waste lagoons, subsurface disposal sites for chemical or low-radioactive wastes, and deep-well injections of toxic chemicals (reviewed in Piver, 1993). In European groundwaters, the most frequently found contaminants are volatile organic compounds from mineral oil (including BTEX, *i.e.* benzene, toluene, ethylbenzene, *o*-, *p*- and *m*-xylene), as well as chlorinated hydrocarbons and heavy metals (EEA, European Environment Agency, 2007). Moreover, polycyclic aromatic hydrocarbons, phenols, cyanides and others are also present, even though to a somewhat lower extent. Disturbingly, the rate at which new contaminations take place is much higher than the rate at which polluted sites can be remediated. For instance, in Europe, potentially polluting activities are estimated to have occurred at nearly 3 million sites, approximately 250 000 of which are already confirmed to require remediation, and the latter number is expected to increase by additional 50% by the year 2025. In comparison, during the last 30 years only 80 000 sites have been cleaned up (EEA, 2007).

Once contaminated, an aquifer not only loses its qualities as a drinking/ irrigation water provider, but also turns into an inhospitable living space for groundwater fauna. Toxic substances act on different levels – from single molecules or cells, to whole organisms, populations and communities, as well as on different spatial scales – depending on the size of the contaminated area. Moreover, toxicity acts on different time scales – either in terms of short-time pulse-exposures (as sometimes occurring in karstic systems and caves, where connectivity and groundwater flow-rate are comparatively high), or in terms of long-lasting contaminations that persist for a long time unless remediation actions are taken (*e.g.* in porous aquifers

with low flow velocities). Depending on these different scales, the consequences for groundwater fauna can be expected to be diverse – ranging from direct acute and chronic toxic effects (*e.g.* mortality, morphological body deformities, effects on growth and reproduction) to indirect effects (*e.g.* starvation due to reduction in feeding activity, a decrease in locomotory activity in search for food, *etc.*). However, in contrast to terrestrial and surface water ecotoxicology, groundwater ecotoxicology is a relatively 'young' discipline and reliable data for groundwater organisms are still scarce. As the natural abundance of stygofauna is typically low and it is not possible to quickly obtain large numbers of test specimens by breeding them in the laboratory, the use of groundwater species as model organisms for routine ecotoxicological screening is regarded as non-feasible (e.g. Notenboom *et al.*, 1999; Mösslacher *et al.*, 2001). Nevertheless, Mösslacher *et al.* (2001) strongly suggested that groundwater invertebrates should further be used at least in fundamental research in order to assess their sensitivity to anthropogenic stressors and thus obtain the knowledge required for the development of appropriate concepts for groundwater risk assessment and for a sustainable groundwater management. In concordance with this, one major focus in groundwater ecotoxicological studies has been to investigate, whether hypogean species differ in sensitivity from their epigean relatives (e.g. Bosnak & Morgan, 1981; Mösslacher, 2000; Canivet & Gibert, 2002; Krupa & Guidolin, 2003; and more recently: Reboleira *et al.*, 2013) and accordingly, whether surface water species can be used as surrogates in the risk assessment of new chemicals with respect to groundwater ecosystems. The outcomes of this research have been contradictory and dependent on the substance and species tested. While the epigean isopod *Lirceus alabamae* has been observed to be 14 times more sensitive towards cadmium and 2 times more sensitive towards zinc than the stygobitic species *Caecidotea bicrenata*, there was no significant difference in the sensitivity of both species with respect to total residual chlorine (Bosnak & Morgan, 1981). The opposite has been reported by Mösslacher (2000), who found a consistently higher sensitivity of the stygobitic species when comparing the sensitivities of related epigean and hypogean species of isopods, copepods and ostracods towards potassium chloride. With respect to pesticides, a comprehensive study has been conducted in 2001 on behalf of the German Federal Environmental Agency

(Umweltbundesamt) by C. Schäfers and coworkers (Schäfers *et al.*, 2001). They observed that in those cases where the toxicants affected anabolic metabolism or activity (*i.e.* for the fungicide Cyprodinil), the groundwater organisms reacted significantly more slowly (by a factor of 5 to 10) than the surface water species. Nevertheless, as a general conclusion, these authors did not find evidence indicating that groundwater organisms might have an inherent higher sensitivity towards pesticides than surface water species. While this work represents one of the few detailed studies available, it is only based on three different pesticides. Regarding other types of pollutants, such studies are completely missing and toxicity data for true groundwater organisms remain scarce and insufficient. This hampers the progress of groundwater ecotoxicology as compared to surface water and terrestrial ecotoxicology and makes the application of integrative tools in environmental risk assessment such as SSD (Species Sensitivity Distributions) difficult (*e.g.* as in Hose, 2005). Therefore, a more profound knowledge on the resistance of different species of stygofauna towards chemicals and chemical mixtures is still needed in order to develop ecologically sound strategies for the conservation of groundwater ecosystems and biodiversity, as well as appropriate law regulations and monitoring programmes (see section 1.3).

1.2.4. Changes in natural temperature regime

Changes in the natural temperature regime of aquifers often occur as a result of artificial recharge with water having a higher temperature than the ambient groundwater or due to thermal energy discharge (respectively withdrawal), that is related to the use of geothermal energy for the cooling and heating of buildings. Regarding recent and upcoming global challenges in the field of energy policy, the latter technology represents a sustainable alternative to conventional heating/ cooling based on non-renewable energy sources. Hence, it has become a popular and constantly growing branch in energy industry. In March 2013, a total of 290,000 shallow geothermal installations were present in Germany (GtV Bundesverband Geothermie, 2013) with several thousand ones being added each year (*e.g.* in 2012, the number of new installations reached 22,000). On a global scale, the trend is similar. However, while being widely regarded as an 'environmentally safe' technology with respect to pollution (*e.g.* Gao *et al.*, 2009), lately the question has

been raised whether the usage of geothermal energy might possibly have other negative effects on the functioning of groundwater ecosystems. The investigation of these effects has begun only recently, with *e.g.* Brielmann *et al.* (2009). An increase in groundwater temperature is known to cause a decrease in oxygen solubility and has been furthermore shown to induce carbonate precipitation (Griffioen & Appelo, 1993), to cause an increased dissolution of silicate minerals (Arning *et al.*, 2006) and mobilization of organic compounds from sediments (Brons *et al.*, 1991), and a decrease in groundwater oxygen saturation (as summarized by Brielmann *et al.*, 2011, *i.e. Publication II* in this thesis). These physico-chemical changes in habitat conditions are in turn expected to affect microbial communities as well as stygofauna. Moreover, geothermal energy usage also introduces temperature fluctuations into a habitat, which would otherwise be characterized by a high thermal stability, with temperature fluctuations as low as ±1 °C throughout the year (Colson-Proch *et al.*, 2010). An increase in temperature leads to higher physiological activity in organisms (*e.g.* locomotory activity, as well as higher oxygen consumption rates) until a critical threshold is reached where this trend gets reversed and eventually, mortality occurs. For example, Issartel *et al.* (2005) reported that stygobitic amphipods of the cold-stenotherm species *Niphargus virei* that were maintained at an elevated temperature of 17 °C showed a nearly 50% higher oxygen consumption rate and an increased mortality rate as compared to specimens maintained at a temperature of 11 °C. Moreover, even for a species with a broader tolerance range (*N. rhenorhodanensis*), serious long-term effects have been observed: a temperature elevation by 6 °C (again resulting in a 17 °C treatment for individuals naturally living at 11 °C), led to the death of 50% of the tested specimens within 3 months (Colson-Proch *et al.*, 2010). For the harpacticoid copepod *Parastenocaris phyllura*, T. Glatzel reported 100% mortality at temperatures between 19 and 22.5 °C after 84 days (Glatzel, 1990).

Connected to the elevation in physiological rates, is also an increase in energy expenditures – as has been observed for the fruit fly *Drosophila melanogaster*, which showed a reduced fecundity under temperature stress (Krebs & Loeschcke, 1994). This effect is expected to be particularly problematic for stygofauna, since food availability is typically poor in groundwater ecosystems and hence, a reduced fecundity would add to the already low reproductive potential of these organisms.

Other effects of elevated temperature that have been described from laboratory studies include: a doubling in ventilatory activity between 14 and 21 °C for *N. rhenorhodanensis* (Issartel *et al.*, 2005), as well as a threefold higher transcription of heat shock protein (HSP70) genes after 1 month of thermal stress at 16 °C as compared to the control specimens which were maintained at a temperature of 10 °C (Colson-Proch *et al.*, 2010).

On the aquifer scale, Brielmann *et al.* (2009) observed a decrease in diversity of the stygofauna community with increasing temperature, possibly emphasizing the sensitivity of individual groundwater invertebrates towards heat discharge. However, the authors interpreted this result with caution as they could not exclude that the high diversity observed in the thermally unaffected wells might have been also (at least partially) related to the proximity of these wells to a river (and hence, to the increased availability of food). Furthermore, Foulquier *et al.* (2011) observed in a field study on a stormwater recharged, thermally influenced aquifer in France that invertebrates were almost totally absent in those areas, where groundwater was characterized by elevated temperature (22 °C) and nearly anoxic conditions, even though the respective sediment supported the highest microbial activity and biomass. They concluded that the trophic interactions between microorganisms and invertebrates were limited by the environmental stresses in this area (oxygen depletion and groundwater warming), thereby impeding the flow of energy through the groundwater food web. While this example does not allow to deduce whether the fauna was absent due to the oxygen scarcity or rather the increased temperature, it nevertheless demonstrates another aspect of temperature elevation effects: since oxygen solubility in water, as well as microbial and faunal respiration activities are related to temperature, changes in natural temperature regime influence the overall availability of oxygen in aquifers.

Despite the studies mentioned above, understanding of temperature effects *in situ* is still insufficient. For example, little is known on the physiological tolerance range of different stygobitic species (other than *Niphargus virei* and *N. rhenorhodanensis*) to temperature elevations and on the question whether groundwater invertebrates can sense temperature changes and actively avoid areas with unfavourable conditions. In case they can, it is further not known,

whether this active migration could be fast enough in order to escape in time – before major adverse effects of temperature stress or even lethality occur. Also, in order to assess and quantify sublethal temperature stress, appropriate analytical methods are required. Most of these questions were addressed within the present dissertation project (see section 2.2).

1.3. *Vulnerability of groundwater ecosystems and their protection through legislation*

Aquifers are considered to be particularly vulnerable against stressors and with a potential for recovery lower than that of other ecosystems, *inter alia* due to the specific life-cycle characteristics of groundwater fauna. Stygobites typically have no resting or dispersal stages, are long-lived, slow-growing and have few offspring (Humphreys, 2009). Once depopulated as a result of disturbance, habitats are hence expected to be recolonized mainly through migration (*i.e.* specimens returning from the surrounding areas) rather than via the reproduction of the few *in situ* survivors. Either way, regardless of the manner of recolonization, groundwater communities can only very slowly recover from reductions in the population sizes of their species. For example, a karstic area in France that was partially depopulated due to a discharge pumping test, had one year later still not recovered its previously present population of harpacticoid copepods (Rouch *et al.*, 1993). Similarly, a groundwater community that had become dominated by polysaprobic surface water oligochaetes (*Tubifex tubifex*) due to regular sewage infiltration only just began to show first signs of recovery one year after the disturbance (in terms of a decrease in oligochaete abundance and first reappearance of stygobitic species). Nevertheless, it was still far from regaining biological equilibrium, so that the invertebrate assemblages were dominated by stygoxene and stygophilic species rather than by stygobites (Malard *et al.*, 1996).
The recolonization of such previously disturbed areas also depends on the dispersal ability of the invertebrates and on the presence or absence of geomorphological migration barriers. Stygofauna are considered to have poor dispersal abilities, based on the observed high proportion of short-range

endemics, the low frequency of sympatry or congeners, the low reproductive potential, the production of relatively large offspring, the long period of brood care, and the lack of easily dispersed stages (as summarized in Humphreys, 2000). Moreover, genetic studies with isopods have shown that each restricted aquifer can have an isolated and phylogenetically unique population (reviewed by Wilson, 2008). Thus, another factor that contributes to the high vulnerability of groundwater ecosystems is their 'special nature of biodiversity' (Humphreys, 2009). As mentioned above, many groundwater species occupy very circumscribed areas (*e.g.*, most inland aquatic isopods are short range endemics) and as a consequence, human disturbances (such as the over-exploitation of water) can pose serious threats to their survival (Wilson, 2008). Notenboom *et al.* (1994) even concluded that the probability of complete species extinction after certain pollution events is high and this is expected to apply similarly to other types of stressors as well. It is therefore alarming that currently, the high vulnerability of groundwater ecosystems seems to be inconsistent with the extent of their protection. This will be briefly illustrated with two examples, corresponding to the two main groups of anthropogenic stressors investigated in this thesis – toxic pollution and changes in natural groundwater temperature regime.

While the European Commission Groundwater Directive (GWD 2006/118/EC, 2006) recognizes groundwater as the 'most sensitive and the largest body of freshwater in the European Union', it only demands the protection of the good chemical and quantitative status of groundwater – contrary to the Water Framework Directive (WFD 2000/60/EC, 2000), where for surface water bodies, also a good *ecological status* is prescribed. In order to define the 'good groundwater chemical status', the GWD requires the EU Member States to derive 'threshold values' for relevant chemical parameters (*i.e.* those parameters that cause a groundwater body to be at risk of failing to achieve good status). For Germany, these threshold values have been derived by LAWA[7] and specified in a report in 2004 (Altmayer *et al.*, 2004). The authors used data from ecotoxicological tests with surface water fauna (algae, microcrustaceans and fish) instead of stygobites and presented the following rationale for this: 1) 'there are currently no standardized

[7] LAWA: 'Bund/Länder-Arbeitsgemeinschaft Wasser', an association of the German ministries for water management and legislation.

ecotoxicological tests for groundwater fauna available', and 2) 'it can be assumed as a first approximation, that groundwater communities are well represented by the sensitivity distribution of surface water species' (referring to the above mentioned study by Schäfers *et al.* (2001) on pesticides, see section 1.2.3). More recently, evidence has been accumulating that due to a number of metabolic differences, surface water species should not uncritically be used as surrogates (Humphreys, 2007). Accordingly, threshold values derived from surface water species are not generally accepted as adequate for the protection of groundwater fauna. Hose (2005) argued that based on the different taxonomic compositions of surface water and groundwater communities (the latter having a higher proportion of crustaceans and in most cases completely lacking algae, plants, insects, as well as fish and other vertebrates), the two communities can be assumed *a priori* to have a differing species sensitivity distribution. In his study, he concluded that surface water quality guidelines may not be adequate for the protection of groundwater ecosystems. Similarly, a study by Notenboom *et al.* (1999) revealed that the drinking water standard in the EU of 0.1 $\mu g\ L^{-1}$ for several pesticides (as defined by the Drinking Water Directive 98/83/EC, 1998) was not low enough in order to guarantee for groundwater ecosystem protection. At present (*i.e.* 14 years later), this value is still in place and it was furthermore included in the Water Framework Directive (WFD, 2000/60/EC, 2000) and the Groundwater Directive, where it is one of the quality standards defining 'good chemical status'. Accordingly, Larned *et al.* (2012) recently pointed out that 'in most nations, current groundwater policies provide inadequate protection for phreatic ecosystems and cannot ensure sustainable groundwater use'. To some extent, this seems to be owed to the scarce knowledge and the still insufficient quantification of the detrimental effects that stressors have on groundwater ecosystems. However, there also seems to be a lack of effective communication between the scientists dealing with groundwater ecology and the water planners and/ or water policy-makers (Danielopol *et al.*, 2004).

Regarding the protection of aquifers against anthropogenic changes in natural temperature regime (*i.e.* the second stressor of concern in this thesis), there are no common regulations on the European level. Operating installations for shallow geothermal energy usage can lead to local temperature anomalies, *i.e.* heat or cold

plumes, which in some cases can be assumed to have adverse consequences for the environment (see section 1.2.4). However, as demonstrated by a study reviewing the legal status of shallow geothermal energy usage in 60 countries worldwide, most of the countries have no legally binding regulations or even guidelines to define groundwater temperature limits for heating/ cooling and minimum distances between geothermal systems (Haehnlein *et al.*, 2010a). Moreover, the effects of temperature changes on groundwater ecosystems are still largely unknown and the knowledge required to define the range of ecologically tolerable temperature alterations is not available yet. Accordingly, the authors of the above mentioned study stressed the 'need for further research on the environmental impact and legal management of shallow geothermal installations' (Haehnlein *et al.*, 2010a).

2. Aims and Methodological Approach

The research performed in this dissertation focused on the effects and implications of two groups of stressors acting on groundwater ecosystems – contamination with toxic chemicals and changes in natural groundwater temperature regime. In particular, the aim was to quantify the impacts of toxic compounds and elevated temperatures on the survival of groundwater fauna. Moreover, sublethal effects of heat stress, as well as the preferred temperature range were assessed for selected stygobites. Where necessary, appropriate methods were developed.

2.1. Toxic stress

Ecotoxicological tests are an important tool for the assessment of toxic stress on aquatic organisms. At the same time, it has been pointed out by several authors that there are no standardized testing procedures available for obligate groundwater fauna and that the presently existing ecotoxicological data are insufficient in order to define regulatory standards for groundwater contaminants based on true groundwater species (see sections 1.2.3 and 1.3). Therefore, one of the main aims of the present dissertation project was to develop an ecotoxicological bioassay for groundwater invertebrates, which particularly takes into account their specific physiological characteristics, *i.e.* the possibly delayed manifestation of toxic effects resulting from their low metabolic rates **(*Aim I*)**. This aim also included the requirement that the bioassay should be suitable for testing the effects of volatile organic compounds (VOCs), since VOCs from mineral oil (including BTEX) and chlorinated hydrocarbons are among the most frequent contaminants in European groundwaters (EEA, European Environment Agency, 2007). The second aim **(*Aim II*)** was to apply this newly-developed bioassay in order to assess the toxicity of toluene (as a model VOC) on the stygobitic amphipod *Niphargus inopinatus*. Toluene was chosen as a model compound due to its ubiquitous occurrence in aquifers: for example, according to a report by the U.S. Geological Survey, toluene is currently among the top five most frequently detected VOCs in USA groundwater (Zogorski *et al.*, 2006).

VOCs are referred to as 'difficult-to-test' substances in ecotoxicological literature (Rufli *et al.*, 1998; OECD, 2000) due to their characteristic physical and (bio)chemical features, *e.g.* volatility, limited solubility in water, biodegradability, as well as sorption and bioaccumulation potential. Therefore, several aspects (specified below) had to be considered during the development of the bioassay. Moreover, the methodological challenges with respect to testing procedures were even intensified by the long duration of the test which was necessary in order to account for the reduced metabolism of the groundwater invertebrates. For example, while the majority of ecotoxicological tests are conducted in open test vessels to allow sufficient oxygen availability, this was not possible with volatile substances. Here, the vials had to be closed tightly, in order to prevent volatilization losses of the test substance and thus, to avoid an underestimation of toxicity. On the other hand, this posed limitations in terms of oxygen availability. By conducting the bioassay in crimp-top glass vials for GC/MS headspace analysis, an approach was chosen that allowed the analysis of toluene concentrations directly in the test vials, as soon as the testing period was accomplished. That way, any volatilization losses were avoided that would have otherwise arisen during sampling of the water and/or animal transfers. At the same time, the air in the headspace compartment contained enough oxygen in order to sustain the life of the amphipods throughout the entire time period of duration (more than 20 days) that was chosen for the test.

The full description of the newly developed bioassay ***(Aim I)*** and the results of the ecotoxicological study with *Niphargus inopinatus* and toluene ***(Aim II)*** are contained in **Publication I**. In brief, the amphipods were exposed to a series of different toluene concentrations and the mortality that occurred as a result of toluene toxicity was recorded at regular time intervals. Apart from the animal and the toxicant solution, each test vial contained a defined amount of water, quartz sand (as a crawling substrate for the amphipods) and air (as a reservoir for oxygen in the headspace compartment). This setup required that the amount of test substance lost to the headspace compartment was calculated precisely in order to obtain the actual toxicant concentration to which the animals were exposed in the aquatic compartment of the vial. The time required for Henry equilibrium to establish in the test vials, and hence, the time required in order to achieve a stable

concentration of the contaminant in the water was assessed in a preliminary experiment and constituted one part of the bioassay development.

Other methodological aspects that were addressed during the method development process included i) the comparison of different schemes for monitoring of the toxicant concentrations during the test, ii) the confirmation that there is a sufficient amount of oxygen present in the vials, and iii) the search for strategies to minimize the biodegradation of toluene during the long duration of the test. Different approaches to cope with the latter problem were tested, including the use of antibiotics to inhibit microbial biodegradation and 'washing' of the test animals before they were introduced into the test vial.

To take into account the possibly delayed manifestation of toxic effects in groundwater fauna, a time-independent (TI-) approach was chosen, *i.e.* 'an acute toxicity test with no predetermined temporal end point which continues until the toxic response has ceased or other (practical) considerations dictate that the test be terminated' (Rand, 1995). Mortality was recorded at short time intervals (daily during the first ten days and later on with a maximum gap of 3 days). The obtained data were evaluated in a dynamic manner (*i.e.* for each day of observation a separate LC_{50} value was calculated) in order to make the test comparable to different tests with surface water invertebrates. At the end of the test, an ultimate LC_{50} was calculated, *i.e.* 'that level of the toxicant beyond which 50% of the population cannot live for an indefinite time', or in other words, the 'concentration which would kill the average [niphargid] on long exposure' (as defined by Sprague, 1969). This ultimate LC_{50} allows the comparison of the sensitivities of species that have different toxicity dynamics in time and thus prevents the problems arising from the different metabolic rates of related groundwater und surface water species.

2.2. Stress due to changes in natural temperature regime

In order to obtain a better understanding of the ecological implications of temperature stress on groundwater ecosystems, it is necessary to assess the impacts of elevated temperatures on the survival of different stygobitic species, as well as the time scales at which elevated temperatures can be tolerated.

Furthermore, the question needs to be considered, whether groundwater fauna can sense areas with elevated or reduced temperatures and if so, whether they would actively avoid areas with unfavourable conditions. These issues were tackled within the second part of the dissertation project (in collaboration with others, see page VIII of this thesis) and accordingly, the following aims and working hypotheses were formulated:

- ***Aim III:*** to assess for selected stygofauna species (amphipods and isopods): i) the temperatures that are lethal for 50% of the tested populations after a certain period of time (*i.e.* the $LT_{50,t}$ value), and ii) the time period for which a certain temperature can be tolerated by 100% of the tested population.

  Hypothesis I: Different groundwater species have different sensitivities towards temperature elevation and are able to tolerate a given unfavourable temperature for different periods of time.

- ***Aim IV:*** to assess whether these groundwater invertebrates are able to sense differences in water temperature and hence, to actively choose their preferred habitat.

  Hypothesis II: the tested groundwater invertebrates can sense areas with different temperatures in a temperature gradient and will actively choose their preferred location. They will avoid areas with temperature extremes, as they are adapted to a quite narrow range of relatively stable water temperatures.

In order to assess the lethal temperature doses, as well as the highest observed tolerable temperatures for the different stygobitic crustaceans (*Aim III*), temperature dose-response studies were performed[8] resembling the dose-response studies which are usually used in the ecotoxicological testing of chemicals. Specimens of the groundwater amphipod *Niphargus inopinatus* and the isopod *Proasellus cavaticus* were tested in comparison. In brief, subgroups of test organisms of each species were exposed to a series of 6 different temperatures ranging from 4 to 24 °C and mortality was recorded after 24h, 48h, as well as daily for a period of several weeks. The mortality data were plotted against temperature and the

[8] The experiments were performed by Kathrin Schreglmann and Francesco Ferraro in their diploma thesis and master thesis, respectively. I was involved in the work as a co-supervisor, see page VIII of this thesis.

resulting temperature-response curves were analyzed according to the procedures usually applied for dose-response curves with toxic chemicals. For each time point of observation, separate curves were fitted in order to obtain a separate LT_{50} value. Further methodological details are given in **Publication II**, as well as in Ferraro (2009) and Schreglmann (2010).

The question whether groundwater invertebrates can sense temperature changes and actively choose their preferred residence location (*Aim IV*) was investigated in an especially designed glass chamber[9] in which a temperature gradient ranging from 1.8 to 36 °C was established. The same two species of stygobitic crustaceans were used as in the temperature dose-response studies. Four test specimens per trial were positioned within that area of the gradient, where the temperature prevailed, to which they had been previously acclimated (~12 °C). Subsequently, the animals were allowed to move freely within the gradient chamber. The position of each animal in the temperature gradient was recorded every 30 minutes during the first four hours of the experiment, and again on the next day, during the last five hours of the experiment (*i.e.* hours 0 to 4, and 19 to 24). Each trial was performed in triplicate and oxygen concentrations were monitored in order to exclude bias resulting from temperature-related differences in oxygen concentrations. The control treatment was performed in the same glass chamber, but without a temperature gradient, *i.e.* the water temperature was equal to 12.7 °C within the entire chamber. At the end of the experiment, the preferred residing location of each species (as corresponding to its preferred temperature range) was determined by selecting those areas, which where visited most frequently by the test specimens. Further details on the experimental setup are included in **Publication II** and Schreglmann (2010).

A third part of the dissertation project[10] focused on the question whether short-term, sublethal temperature elevations are sufficient to cause physiological stress in stygofauna and whether this is reflected by a change in the catecholamine levels of the animals. Catecholamines (*i.e.* noradrenaline, adrenaline, and dopamine) are substances that are known to be involved in the stress response of many

[9] The gradient chamber was designed and constructed by Christian Griebler and Günter Teichmann, Institute of Groundwater Ecology, Helmholtz Zentrum München.

[10] These investigations were performed in collaboration with partners from the department Molecular Exposomics at HMGU, the German Research Center for Environmental Health (see page VIII of this thesis).

organisms (vertebrates, as well as invertebrates), but their physiological functions in invertebrates are not yet fully understood (Lacoste *et al.*, 2001; Aparicio-Simón *et al.*, 2010). Moreover, the way catecholamines act has been observed to be species-specific and to vary between different crustacean taxa (see introduction within Publication IV). With respect to stygofauna, at the beginning of the present project, no research on catecholamines had been reported yet, and even the presence of catecholamines in stygobitic taxa had not been demonstrated. Therefore, ***Aim V*** was to investigate, whether adrenaline, noradrenaline, and dopamine do actually occur in the stygobitic amphipod *Niphargus inopinatus* and if so, whether the amounts are high enough in order to be detected in single specimens. As the presence of catecholamines in niphargids could be demonstrated successfully (see **Publication III**), two further questions were addressed: (i) whether catecholamine levels reflect temperature stress in stygobitic amphipods and (ii) whether physiological differences in terms of temperature stress response exist between related surface water and groundwater amphipods – as a result of their respective adaptations to the different temperature regimes in both habitats. While surface water amphipods in temperate regions are exposed to (and are able to tolerate) frequent seasonal, as well as diurnal fluctuations in water temperature, groundwater amphipods experience quite stable temperatures throughout the whole year. Accordingly, ***Aim VI*** was to compare the catecholamine levels in two related amphipods from different habitats (*i.e. Gammarus pulex* from surface streams, and *Niphargus inopinatus* from a porous aquifer) and to assess their immediate response towards a sudden, short-term, sublethal temperature elevation. The hypothesis formulated was, that the catecholamine levels of the amphipods will change in response to the change in temperature. Furthermore, since *N. inopinatus* is adapted to relatively stable water temperatures, this species was hypothesized to be more sensitive towards sudden, short-term, sublethal temperature elevations than *G. pulex*. This was expected to be reflected by a pronounced difference in the catecholamine levels of temperature-stressed *versus* non-stressed niphargids, whereas the difference in gammarids was anticipated to be smaller, or even negligible ***(Hypothesis III)***.

In order to test this hypothesis, pre-adapted specimens that were kept at a stable water temperature of 12 °C in the laboratory, were exposed to sudden, short-term

temperature elevations of either +6 °C, +12 °C, or to no elevation at all (in the controls). After the temperature elevation, the amphipods were shock-frozen in liquid nitrogen. Since the niphargids were too small for hemolymph extraction, each animal was homogenized and the catecholamine concentrations were analyzed from whole-animal extracts. A detailed description of the study is included in **Publication IV**.

In summary, with respect to anthropogenic temperature modifications, several different aspects were given attention in this dissertation – ranging from the direct lethal effects of temperature elevations on groundwater crustaceans (**Publication II**) to short-term physiological stress (**Publications III and IV**) and the choice of preferred habitat as related to the temperatures required by a specific organism (**Publication II**).

3. Results and Discussion

Maintenance of biodiversity is a critical test of whether anthropogenic water use or ecosystem modifications are sustainable (Dudgeon et al., 2006). Conversely, the loss of species under the action of an anthropogenic stressor reflects that the magnitude of the stressor (or its direct and indirect consequences) have exceeded the species' critical threshold of survival. Alarmingly, on a global scale, freshwater ecosystems are experiencing serious biodiversity declines (Dudgeon et al., 2006). Extinction rates of freshwater animals in North America, based on combined data sets for unionid mussels, crayfish, fishes and amphibians have been predicted to reach 4% per decade in the recent century, (*i.e.* five times higher than the species losses calculated for terrestrial habitats), if no effective conservation actions are undertaken (Ricciardi & Rasmussen, 1999). Regarding groundwater fauna, such large-scale studies are still missing. However, exemplary reports from single sites affected by anthropogenic stressors already indicate that the numbers might be similarly high. For instance, dramatic loss in stygobitic species occurred due to artificial recharge with municipal effluent from wastewater treatment plants in Southern Italy: a dataset spanning a sampling period of 60 years (from 1945 to 2004) revealed that 42.1% of the species originally present, could not be detected anymore once the artificial recharge activities had started in 1991 (Masciopinto et al., 2006). Although this number applies only to a local scale and is not directly comparable with the global extinction of species, it would correspond to an extinction rate of 7.1% per decade for this particular site. Considering the exceptionally high percentage of short range endemic species in groundwater, it is very likely that in some cases, local extinction can be directly translated into global extinction (Notenboom *et al.*, 1994; Humphreys, 2009).

Due to the lack of information on the role of stygofauna, as well as species richness for the functioning of groundwater ecosystems (see section 1.1), the implications of species loss on the stygobitic communities, on the key processes in the ecosystem, as well as on the provision of ecosystem services, cannot be realistically assessed at this state. What is more, even the basic knowledge on how

anthropogenic stressors affect groundwater organisms is still scarce. On the other hand, such knowledge is required in order to provide a solid basis for the development of ecologically sound and sustainable groundwater management strategies by public authorities. The research performed in this thesis was designed to contribute to a better understanding of the impacts of toxic, as well as temperature-related stress on groundwater biota, and to the assessment of stressor levels that are critical for their survival.

3.1. Toxic stress

In the first part of the dissertation project, a new bioassay for testing the ecotoxicological effects of volatile organic compounds (VOCs) on stygobites was developed. The protocol takes into account both the methodological aspects resulting from testing the effects of VOCs, and the reduced metabolism of groundwater invertebrates. The volatility of the test substance was addressed in a 'closed from start to analysis'- design, while the possibly delayed manifestation of toxic effects in the 'slowly metabolizing' groundwater fauna was taken into account by using a time-independent (TI-) approach. With the development of this bioassay, and the exemplary assessment of the toxicity of toluene for the stygobitic amphipod *Niphargus inopinatus*, the first two aims of the project (*Aims I* and *II*) were accomplished (see *Publication I*). The results of the bioassay revealed that the toluene levels that are lethal for 50% of the tested individuals of *N. inopinatus* can indeed be considered as being of high environmental relevance. For example, the ultimate LC_{50} of toluene for *N. inopinatus* was estimated at 23.3 mg L^{-1}, which is well within the range of concentrations that have been reported from contaminated field sites (e.g. Stelzer *et al.*, 2006; Anneser *et al.*, 2008; Winderl *et al.*, 2008; Yagi *et al.*, 2009; Anneser *et al.*, 2010). Moreover, the new bioassay can be easily adapted for the use with other groundwater species/ volatile substances, so that it can serve as a tool for collecting further ecotoxicological data with true stygobites. This will allow obtaining of a broader knowledge on the sensitivity of groundwater fauna towards chemical contamination and – on the long term – will serve as a better basis for the derivation of groundwater quality standards as required for groundwater ecosystem protection.

To our knowledge, at present there are no other ecotoxicological studies available that have tested the effects of toluene (or other BTEX-compounds) on groundwater invertebrates. In contrast, several studies with surface water fauna, particularly crustaceans, have been performed since the 1980s, *e.g.* with the amphipod *Gammarus minus* (Horne & Oblad, 1983), the cladocerans *Daphnia magna* (Bringmann & Kuhn, 1977; LeBlanc, 1980; MacLean & Doe, 1989) and *Ceriodaphnia dubia* (Marchini *et al.*, 1993; Niederlehner *et al.*, 1998), as well as with the copepods *Cyclops viridis* (Panigrahi & Konar, 1989) and *Diaptomus forbesi* (Saha & Konar, 1983) – as listed in the US EPA Ecotox database. The reported LC_{50} values for these species, with observation durations between 1 and 4 days, range from 9 to 470 mg L^{-1} toluene, although it can be assumed that the latter value is probably an overestimation since it marks the solubility limit of toluene and the test vials were not kept closed in order to minimize volatilization losses. In a similar line of argument, *i.e.* due to methodological drawbacks, several of the above mentioned studies were later considered 'invalid' by the European Chemicals Bureau in the EU Risk Assessment Report for toluene (Hansen *et al.*, 2003). Out of the studies that were considered as 'valid' in the report, a much narrower range of $LC_{50,\ 24\text{-}96h}$ values remains, which lies between 3.78 (Niederlehner *et al.*, 1998) and 14.9 mg L^{-1} for daphnids (Adema, 1991), and reaches up to 74.2 mg L^{-1} (Potera, 1975) when other crustaceans (marine and freshwater) are included. In comparison, the LC_{50} values for *N. inopinatus* observed in our study during the first 4 days of exposure are in the same order of magnitude, ranging from 49.6 to 140.9 mg L^{-1} (see Table 2 in *Publication I*). However, it has to be noted that due to the putatively slower metabolism and the possibly delayed manifestation of toxicity effects in groundwater organisms, pronounced differences in the toxicity dynamics in time might be present between surface water and groundwater species (discussed in detail in *Publication I*). Therefore, such comparisons of LC_{50} values should only be interpreted with care. Instead we suggest that in future, more often ultimate LC_{50} values should be compared, in order to obtain a more realistic evaluation of the sensitivities of groundwater and surface water invertebrates.

Another aspect that should be considered is the problem of how to upscale the significance of laboratory findings obtained from the observation of single individuals to the situation *in situ*, *i.e.* to the population, the community and the

ecosystem level. While this is a common issue in all eco(toxico)logical investigations, it can be argued that it is particularly difficult with respect to groundwater ecosystems. There are several reasons for this, including *inter alia* i) the slow reproduction of stygofauna, which limits the availability of large numbers of organisms for comprehensive studies, ii) the slow growth of stygobitic animals, which makes the assessment of sublethal effects such as growth rates, fecundity rates, numbers of offspring, *etc.* extremely difficult, and iii) the poor accessibility of groundwater ecosystems, which mostly allows ecologists to obtain a quite restricted view depending on the number of monitoring wells available. These factors pose a great challenge to the relatively 'young' scientific field of groundwater ecotoxicology and limit the direct transfer and application of surface water concepts as will be shortly illustrated with the help of two examples.

One approach that is frequently used for ecological risk assessment and the derivation of environmental quality criteria in surface water ecosystems it the construction of Species Sensitivity Distribution (SSD) curves (Posthuma *et al.*, 2001). This approach accounts for the fact that different species possess different sensitivities towards a certain toxicant. The toxicant level which is hazardous only to the most sensitive species is estimated based on the statistical distribution of the different sensitivities (van Straalen, 1994). The SSD approach was applied to groundwater ecosystems some years ago by Hose (2005). However, due to lack of sufficient ecotoxicological information on groundwater species, the author had to use data from surface water surrogate species for the calculations – a solution which was inadvertently associated with drawbacks, as later commented by Humphreys (2007).

Another approach used in surface water ecotoxicology to extrapolate the toxic effects measured under standardized test conditions with single individuals to the population level, is individual based population modeling. For example, such a model was developed by (Preuss *et al.*, 2010) in order to estimate the population-level effects of 3,4-dichloroaniline (3,4-DCA) on the water flea *Daphnia magna*. Using (i) dose-response data from standard ecotoxicological tests describing the effects of 3,4-DCA on the mortality and reproduction of individual water fleas, as well as (ii) data from laboratory experiments with different amounts of food, the model was able to predict the extinction probability of a daphnid population

under environmentally relevant conditions (see Preuss *et al.*, 2010). The study also showed that the daphnid populations were more prone to extinction when food availability was low. Considering that food scarcity is a typical characteristic of aquifers (see section 1.1), such an increased sensitivity towards chemical toxicity under poor food availability may have serious implications for stygofauna. On the other hand, it can also be argued that this effect might be only of minor importance to groundwater species, due to their physiological adaptations to withstand starvation (Hervant *et al.*, 1997; Hervant *et al.*, 1999; Mezek *et al.*, 2010). While the development of such individual-based models for stygobitic species would provide us with important new insights for the ecological risk assessment in aquifers, this progress is still hampered by the physiological limitations posed on research by stygobites (*e.g.* slow growth and reproduction as mentioned above) and hence, by the currently existing lack of data as required by such models.

The two examples demonstrate that many useful concepts that have been developed and successfully applied to surface water ecosystems may not be readily transferrable to the organisms living in groundwater. Therefore, the development of alternative, suitable concepts for environmental risk assessment in groundwater ecosystems, as well as the extension of the currently scarce ecotoxicological knowledge (see section 1.2.3) is urgently needed. If adopted within future studies, the new bioassay that was developed during the course of this dissertation project will hopefully make a contribution.

Despite the challenges mentioned above, there is still a vast potential for the further development of groundwater ecotoxicological studies, for example based on the behaviour of stygofauna. It is still unknown, whether stygofauna can sense contaminants and actively move against groundwater flow direction in order to escape. Furthermore, it is not known whether sublethal exposure to toxicants leads to a decrease in feeding activity and consequently, to a decrease in energy acquisition for groundwater invertebrates, as has been shown for a variety of species (crustaceans, as well as molluscs) and stressors in surface water habitats (reviewed by Maltby, 1999). As bacterial biomass has been shown to be higher at the fringes of a BTEX plume (Anneser *et al.*, 2008), while at the same time, the contaminant concentrations were lower, it can be hypothesized that bacteria located in the plume fringes might represent a potential food source for

stygofauna. If the invertebrates are able to withstand contaminant toxicity for short periods of time, they might temporarily enter the contaminated zones for feeding and furthermore, they could potentially influence the contaminant degradation rates of the bacteria through their grazing and bioturbation activities (see section 1.1). The ultimate LC_{50} value of 23.3 mg L^{-1} toluene that was estimated for *Niphargus inopinatus* within this dissertation thesis is similar to the toluene concentrations that were measured by Anneser *et al.* (2008) at the plume fringes of a BTEX plume near Düsseldorf, Germany. Hence, it can be speculated that theoretically at least some of the amphipods in a population might be able to withstand toluene toxicity and enter the contaminated zones to feed on the bacteria. This scenario is probably not very likely, since it has to be considered that contaminant plumes often comprise mixtures of toxicants, so that their combined toxicity can be expected to be higher. Moreover, as mentioned above, the amphipods might exhibit a reduced feeding activity or even actively avoid the contaminant. Nevertheless, in a different setting, with lower contaminant concentrations, such temporary invertebrate grazing on contaminant degrader communities seems conceivable, considering the else limited availability of food in aquifers. A comparable situation has been observed in the Ayyalon cave in Israel, where stygobitic crustaceans live in the redox interphase between oxygenated and anoxic water and enter the anoxic waters in order to feed on the thick mats of chemosynthetic sulfide-oxidizing bacteria (Por, 2007). Without doubt, whether such feeding behaviour can really occur in contaminated aquifers, and hence – whether groundwater invertebrates are able to actively contribute to the contaminant degradation processes *in situ* via grazing and bioturbation, remains to be tested in future studies.

3.2. Stress due to changes in natural temperature regime

3.2.1. Survival at elevated temperatures

In line with *Aim III*, several LT_{50} values for selected species of groundwater isopods and amphipods were determined, *i.e.* those temperatures that are lethal for 50% of the tested specimens after a given period of time. Additionally, it was

investigated for how long certain elevated temperatures can be tolerated by the entire tested population. For the amphipod *N. inopinatus*, the $LT_{50,24h}$ (± standard error) was equal to 27.1 °C (± 0.5), and for the isopod *P. cavaticus* the same parameter was equal to 23.0 °C (± 0.1). Relatively soon, after five days of exposure, the values dropped to reach an $LT_{50,5d}$ of 23.3 °C (± 2.9) for *N. inopinatus*, and 16.6 °C (± 3.8) for *P. cavaticus* (details are given in *Publication II*). While these results already indicate a slightly higher susceptibility to thermal stress in the isopods, the difference between the two crustacean species becomes even more distinct when the temperatures tolerated by the entire tested group of each species are compared. The highest temperature that could be tolerated by all isopods for 24 hours was 16 °C, while the amphipods tolerated 20 °C. Moreover, the amphipods endured being exposed to 20 °C for a total of 20 days without a single animal dying. In contrast, in the isopod group, the same temperature led to 20% mortality within 24 hours of exposure. The highest temperature treatment that could be tolerated by all isopods and for the longest time was 12 °C for 5 days in our study. The highest temperature that was tolerated by all amphipods for the longest time was 16 °C (for > 50 days).

Our findings for *N. inopinatus* (in particular, 50% mortality at 20.7 °C after 25 days) are consistent with the observations of Issartel *et al.* (2005) in other *Niphargus* species. These authors reported 50% mortality for the stenothermal species *N. virei* at a temperature of 21 °C within 8.9 days, while half of the tested population of the eurythermal species *N. rhenorhodanensis* survived this same temperature for 111.6 days. The thermal tolerance of *N. inopinatus* observed in our study is therefore located between the tolerance ranges of the two species, but closer to the one of the stenothermal species *N. virei*.

With respect to *Hypothesis I* which predicted that different groundwater species will have different sensitivities towards temperature elevation and will be able to tolerate a given unfavourable temperature for different periods of time, two different outcomes were possible. On one hand, assuming that stable temperatures would lead to a narrowing of temperature tolerance range, it could have been that different groundwater species have the same temperature tolerance range since they are naturally exposed to the same, quite stable water temperatures with little fluctuations throughout the year. On the other hand, it was also conceivable that

different stygobitic species would have different tolerance ranges as they might have had different life histories, might have been inhabiting the groundwater realm for different periods of time (according to their time points of groundwater colonization), or as they might have had different rates of evolutionary adaptation. The former assumption (that stable temperatures result in a narrow tolerance range) corresponds to the classical evolutionary hypothesis which also predicts that tropical ectotherms (which are exposed to a low thermal variance throughout the year) should be physiologically adapted to a narrower temperature range than temperate-zone ectotherms, which experience a broader, more variable range of temperatures (van Berkum, 1988). This theory has been exemplarily tested by F. H. van Berkum (1988) on lizards and the results of the study showed that the tolerance ranges of the tropical lizards were indeed narrower than those of the temperate-zone lizards. However, at first unexpectedly, their performance breadth, *i.e.* the temperature range in which the animals performed best (in terms of sprint speed) was not consistently narrower than the one found in temperate-zone lizards and hence, did not support the hypothesis. The author attributed this to the ability of the temperate-zone lizards to actively choose zones with favourable temperatures when they are active in the field and thus perform thermoregulation by means of their behaviour. As a result, their body temperature variability did not reflect so much the variability of the temperatures in their environment and hence, was independent from latitudinal patterns (van Berkum, 1988). In deep, thermally undisturbed aquifers, there are no "shady" or "sunny" areas that would allow the organism to actively choose between zones with different temperatures. Therefore, it can be expected that the naturally low variability in groundwater temperatures will be acting directly upon the organisms as a driver towards narrowing their tolerance range and will not be 'counterbalanced' by means of behaviour. In the study of Issartel *et al.* (2005), considerable differences in the temperature tolerance ranges of *N. virei* and *N. rhenorhodanensis* were observed (as described above), which would contradict the classical evolutionary hypothesis. As an interpretation, the authors suggested that the broad, eurythermal tolerance range of *N. rhenorhodanensis* (including the species' tolerance to cold) might be a relict adaptation that enabled the survival of this species during glaciation periods in the Pleistocene. Another contradiction to

the classical hypothesis was also reported by Colson-Proch *et al.* (2009), who observed a great variability in the thermal response of different populations within the same species (*N. rhenorhodanensis*), even though all populations had been exposed to similar (and stable) temperature conditions in their habitats.

In our study, the higher susceptibility of *P. cavaticus* towards temperature elevations as compared to *N. inopinatus* also contradicted the expectations derived from the classical evolutionary hypothesis (*i.e.* a similar and narrow temperature tolerance range due to an exposure to similar and stable temperatures). Instead, *Hypothesis I* of this thesis was supported, demonstrating that different groundwater species have different sensitivities towards temperature elevations, as well as a different capability of withstanding elevated temperatures in time. Together with the two studies mentioned above (Issartel *et al.*, 2005; Colson-Proch *et al.*, 2009) our results indicate that in groundwater ecosystems, apart from temperature stability, also other factors such as climatic history have to be taken into account for the interpretation of species-specific temperature tolerance patterns.

Another aspect of our results that has to be considered is their implication with respect to the operation of shallow geothermal installations. If groundwater temperatures near a shallow geothermal installation are modified by a maximum of ±6 °C as currently recommended in Germany (VDI, 2000), this may result in groundwater temperatures below 4 °C in winter and above 20 °C during summer (see Brielmann *et al.*, 2011, i.e. Publication II in this thesis). The results of our temperature dose-response studies show that a temperature of 20 °C cannot be tolerated for a long time by any of the two tested species and will eventually lead to at least partial mortality within their populations. Such mortality can have several consequences, including not only biodiversity losses, but also impairment of groundwater ecosystem functioning, and thus, a potential loss of ecosystem services such as the provision of clean drinking water (see section 1.1.). With respect to obligate groundwater fauna, once a habitat is depopulated, it may take a very long time until its communities are restored – if at all – and hence, until the key processes and functions of the ecosystem are regained (section 1.3).

The potential ability of stygobites to escape a waterborne threat like toxicants or temperature change can differ depending on the groundwater flow velocity, as well as on the crawling speed of the organisms and the average number of hours

per day that they spend actively moving. For example, based on data compiled from previous studies (Schminke, 1997; Weber, 2008; Ferraro, 2009; Haehnlein *et al.*, 2010b), K. Schreglmann calculated that in theory niphargids moving with an average velocity of 4 cm h^{-1} through coarse sand for a total duration of five hours per day (while resting for the remaining 19 hours), might indeed be able to escape the progressing front of a temperature plume which brings along a temperature change of ± 1 °C (Schreglmann, 2010). However, this would only hold true in sandy aquifers with an average flow velocity of 13 cm day^{-1}, while in gravel aquifers with flow velocities of 70 to 100 cm d^{-1} or even higher (Haehnlein *et al.*, 2010b), this would not be possible anymore. This being only a rough estimation, it is nonetheless sufficient to illustrate that the temperature alterations occurring near shallow geothermal installations might indeed pose a threat of high relevance to the survival of the species studied in this thesis. However, in order to predict realistically the stygobites' potential for escaping from stressors, additional studies are needed with respect to the crawling speed and dispersal potential of the invertebrates, their diurnal activity patterns, as well as their likeliness to move actively and unidirectionally away from a stressor.

Based on the results of the temperature dose-response studies, some initial suggestions can be expressed for the development of ecologically sound and sustainable groundwater management strategies. Regarding groundwater biodiversity conservation, Malard *et al.* (2009) showed that a high proportion of groundwater species in Europe can be protected by focusing conservation efforts on a few aquifers distributed in distinct regions. Therefore, in known biodiversity hotspots or in areas inhabited by sensitive stygobitic species such as the one observed in the present project (*Proasellus cavaticus*), protected areas with no temperature alterations should be designated. Ideally, as proposed by Danielopol et al. (2003), groundwater ecosystems should be included in the plans for the designation of protected areas and nature reserves. Moreover, the implemented protection measures should also be extended to consider temperature alterations. That this claim is not unrealistic can be seen in Western Australia, where stygofaunal communities have been included in the list of priority ecological communities, as well as in the list of threatened ecological communities endorsed by the Minister for the Environment (Department of

Environment and Conservation, 2013). For Europe, strategies for the selection of reserve areas for the conservation of groundwater biodiversity (Michel *et al.*, 2009), as well as criteria for the site prioritization required for the protection of rare subterranean species (Danielopol *et al.*, 2009), have been also already developed.

As the specific consequences of stygofauna species' loss for drinking water production are largely unknown, particularly in those areas where drinking water is abstracted, groundwater ecosystem protection should be exclusively based on the precautionary principle and groundwater temperature alterations should be kept at minimum. In all remaining areas, for the present state, as long as there are no legally binding limits for groundwater warming defined yet, the threshold of 20 °C should not be exceeded. Where it is not possible to keep groundwater temperatures permanently below this level as well as for the future planning of new geothermal installations, for the sake of biodiversity conservation and the protection of unique individual species, as well as in order to prevent a potential loss of groundwater ecosystem functions, the performance of environmental impact assessments as it is already prescribed in Western Australia (in accordance with the Environmental Protection Act 1986) should be also considered in Germany (see *e.g.* Environmental Protection Authority, 2003).

Certainly, additional studies are needed in order to broaden our knowledge on the sensitivities of stygofauna towards temperature alterations – not only on the individual, but also on the community level. Particularly, the sublethal and long-term effects of elevated temperatures on groundwater fauna in terms of food searching behaviour and food uptake efficiency, fecundity, *etc.* are worth exploring. In future, such studies will allow scientists to make more precise predictions on the behaviour of the ecosystem, the critical thresholds, and the response of the communities towards temperature stress. Ideally, this would allow a 'loosening' of the precautionary principle in favour of an even wider implementation of geothermal energy usage, while at the same time keeping this important new technology ecologically safe.

3.2.2. Preferred temperature ranges

In order to assess whether groundwater invertebrates are able to sense differences in water temperature and hence, to actively choose their preferred habitat (*Aim*

IV), stygobitic amphipods and isopods were exposed to a temperature gradient and allowed to move freely according to their preference. It was hypothesized that the invertebrates will be able to sense areas with different temperatures and will actively choose a preferred location in order to avoid areas with (unfavourable) temperature extremes (*Hypothesis II*).

Both tested species, the amphipod *Niphargus inopinatus* and the isopod *Proasellus cavaticus*, showed a statistically significant preference for certain areas within the temperature gradient (see Fig. 3 in the text below and *Publication II*). In contrast, in the control treatments, where the temperature was the same throughout the entire experimental chamber, no such preferences were found. This behaviour supported the hypothesis, demonstrating that both species were capable of perceiving the different temperatures and choosing their preferred location. Regarding the active avoidance of unfavourable temperatures, the outcome of the experiment is not so evident, as will be discussed in detail below.

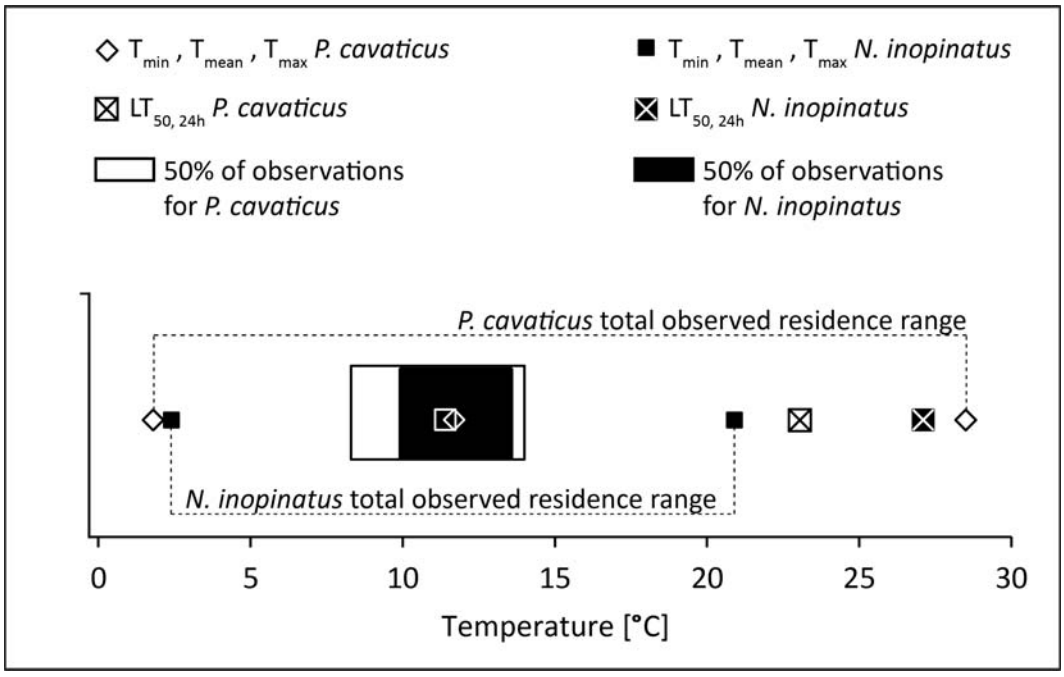

Figure 3: Preferred temperature ranges of the amphipod *N. inopinatus* (filled symbols) and the isopod *P. cavaticus* (empty symbols) in a temperature gradient ranging from 1.8 to 36 °C. The behaviour of each species was observed in a separate experiment. The dotted lines show the entire range of temperatures that were visited by either species. The boxes in the middle of the plot represent the interquartile range (Q_3-Q_1) and show the temperature range where 50% of the observations were made, *i.e.* the range of the most frequently visited temperatures. The LT_{50} values were assessed in temperature dose-response experiments in a separate study (see section 3.2.1).

The mean temperature of all observed spots of residence did not differ much between the two species, being 11.4 °C for *P. cavaticus* and 11.7 °C for *N. inopinatus*. Regarding the total temperature range where specimens could be observed, as well as their preferred temperature range (*i.e.* the temperature range where 50% of the observations were made), both ranges were broader for *P. cavaticus* than for *N. inopinatus*. However, while the $LT_{50,24h}$ of *N. inopinatus* was located outside the total range of observations, indicating that the amphipods were actively avoiding areas with harmful temperatures, this was not the case for *P. cavaticus*: the isopods were also found to reside at temperatures, which would have led to mortality in case of longer exposure.

Assuming that the temperature range chosen by an animal is correlated to the temperature range that it can tolerate best, the observed broader preference range of the isopods might lead to the conclusion that *P. cavaticus* can also tolerate a broader range of temperatures than *N. inopinatus* (especially with respect to higher temperatures). However, this seems unlikely, considering that in the dose-response study *P. cavaticus* was found to experience higher mortalities at elevated temperatures than *N. inopinatus* (see section 3.2.1). Instead, it seems that once the isopods entered an area with highly unfavourable temperatures, they were sometimes not able to actively leave the place anymore. This would be supported by the fact that individual isopods were observed to become immobile and fall into heat torpor at temperatures of 22.9, 23.5, and 25 °C. Disorientation is a typical symptom of heat damage that is being assessed as a response to stress in studies of the thermal resistance in crustaceans (Rodríguez *et al.*, 1996). In experiments with increasing temperatures, this symptom precedes the point of the critical thermal maximum (CTMax), which is defined as 'the thermal point at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death' (Cowles & Bogert, 1944). It seems conceivable that in certain areas of the gradient chamber the temperatures were high enough in order to cause a state of disorientation in the isopods. Such a behaviour did not occur with the amphipods in our study. In contrast, one specimen was even observed to turn around sharply and move on towards colder areas, once it had reached a temperature of 19.8 °C (Schreglmann, 2010). Hence, it seems that disorientation occurred only in *P. cavaticus*, resulting in an artificial

broadening of the total range of observations and consequently, the 'preferred' temperature range of this species. Based on the combined results of the dose-response-study and the gradient experiment, it can be therefore concluded, that the isopods were more susceptible towards elevated temperatures than the amphipods – in spite of their seemingly broader preference range.

3.2.3. Sublethal temperature effects

In order to predict how environmental changes affect biological systems, an understanding is needed of how such changes will impact not only the survival of single individuals, but also the species' reproductive success (Zizzari & Ellers, 2011). However, as breeding groundwater invertebrates in the laboratory has been mostly unsuccessful yet (but see Glatzel, 1990), investigating the effects of stress on the reproductive parameters of stygobitic populations has been impossible for groundwater ecologists. Instead, other sublethal endpoints have been assessed in order to quantify temperature-related and also other types of stress, including changes in respiratory/ ventilatory activity (Hervant *et al.*, 1995; Hervant *et al.*, 1997; Issartel *et al.*, 2005), locomotory activity (Hervant *et al.*, 1997; Ferraro, 2009), energy reserves (Hervant *et al.*, 1999; Hervant *et al.*, 2001), electron transport system activity (Simčič & Brancelj, 2006), and the induction of HSP70 transcription (Colson-Proch *et al.*, 2010).

For the purpose of tracking the immediate stress responses of groundwater invertebrates on the physiological level, the focus of the last part of the dissertation project was set on a different approach – the investigation of temperature stress effects through the analysis of catecholamine levels. To this end, it was first necessary to develop an appropriate method for the detection of catecholamines in single specimens of stygobitic invertebrates and consequently, to investigate whether adrenaline, noradrenaline, and dopamine actually occur in these organisms (*Aim V*). As the niphargids are very small (average body length: 3.8 mm), the catecholamines were extracted from the whole animal rather than from specific parts of the nervous system or the hemolymph. Through the use of two independent analytical methods, HPLC/ EcD[11] and UPLC/ TOF-MS[12], our

[11] HPLC/ EcD: high-performance liquid chromatography coupled with an electrochemical detector.
[12] UPLC/ TOF-MS: ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry.

results confirmed that catecholamines do exist in stygobitic species – not only in the amphipod *Niphargus inopinatus* (see *Publication III*), but also in the isopod *Proasellus cavaticus* (Wang, Z. & Schramm, K.-W., unpublished data). In *N. inopinatus*, out of the three investigated catecholamines, the whole-body concentrations of dopamine (DA) were highest, ranging from 69.3 to 31,835 pg mg^{-1} fresh weight, with an average value of 16,400 pg mg^{-1} FW. The noradrenaline (NA) concentrations ranged from 31.7 to 1,200 (on average 533) pg mg^{-1} FW, whereas adrenaline (A) ranged from 17.7 to 4,443 (on average 314) pg mg^{-1} FW. While DA and NA were detected in all analyzed specimens, adrenaline could not always be found. Nevertheless, when present, the concentrations of all three substances were high enough to be detected in single individuals, thus putting aside the necessity to analyze several pooled specimens per sample. This result was an important prerequisite in order to allow further investigations and comparisons of catecholamine-concentrations on the individual level.

The interspecific comparison between the whole-body catecholamine contents of *N. inopinatus* and *Gammarus pulex*, a related surface water amphipod (*Aim VI*), revealed pronounced and statistically significant differences between the two organisms with respect to all three compounds ($p < 0.05$, Kruskal-Wallis rank sum test). In the niphargids, the average DA levels were roughly 1500 times higher than in the gammarids (pooled data from the whole dataset, regardless of temperature treatments). Similarly, the average NA levels were 195 times higher, and the A levels were 9 times higher in *Niphargus* as compared to *Gammarus*. For example, while the average DA level in *N. inopinatus* was 20,881 pg mg^{-1} FW (lying well within the above described range of values from our first investigation), in *G. pulex* it was only 14 pg mg^{-1} FW. Such large differences were unexpected and considering that groundwater fauna live in an energy-limited environment, the question arose why such high amounts of biogenic amines are present in niphargids. While the DA levels of *N. inopinatus* were comparable to the ones found in another stygobite, the isopod *Proasellus cavaticus* (Wang, Z. & Schramm, K.-W., unpublished data), the feature of having high DA amounts does not seem to be restricted only to the groundwater habitat. For example, similarly high DA levels have been also reported from the fruit fly *Drosophila virilis* (Rauschenbach *et al.*, 1997). On the other hand, the comparatively lower DA

content of *G. pulex* was similar to the one reported for the cladoceran *Daphnia magna* (Ehrenström & Berglind, 1988).

While most of the studies that deal with biogenic amines in invertebrates have focused on the concentrations of catecholamines in the hemolymph or in specific organs, the number of studies reporting whole-animal catecholamine amounts is quite limited. Therefore, at the present state of knowledge, the explanation for these large interspecific differences cannot be readily deduced. Nevertheless, several interpretations are possible (discussed in detail in *Publication IV*). For example, a food-based limitation in regular supply with the essential amino acid L-phenylalanine, which is required for the biosynthesis of catecholamines, might force stygofauna to keep high amounts of catecholamines, and especially DA (the precursor of NA and A), in store. Moreover, storage of high amounts of DA might help the organisms to circumvent the rate-limiting step in catecholamine biosynthesis – the hydroxylation of L-tyrosine to L-DOPA, which is the direct precursor of DA. Further possible interpretations include a potential involvement of DA in the slowdown of reproductive functions in stygobites, their aggressive behaviour (*e.g.* exhibited during cannibalism), or even their residing at an earlier, more primitive stage of evolution as compared to the gammarids (see discussion section in *Publication IV*).

Even though our first study had demonstrated that catecholamines are present in groundwater crustaceans, at that time point the results did not allow any conclusions on the physiological functions of the compounds and whether they are involved in the organisms' response to stress. There was however, one outlier in the dataset (see sample 1 in *Publication III*) which had quite low DA (79 pg mg^{-1} FW) and NA (88 pg mg^{-1} FW) levels and exceptionally high A levels (4363 pg mg^{-1} FW) as compared to all other samples. As a decrease in DA and an increase in A hemolymph concentrations in response to heat stress has been reported from the mollusc *Chlamys farreri* (Chen *et al.*, 2008), it seemed possible that the outlier in our dataset might have been represented by a stressed animal. We assumed that if this was indeed a stress reaction, it could have been caused by short handling stress due to unintended slight agitations of the vial during transfer into liquid nitrogen for shock-freezing. Low DA levels had also occurred in two more niphargids in our dataset that had been observed to swim up to the

water surface just immediately before shock-freezing in liquid nitrogen and were assumed to have also been subject to slight mechanical disturbance. These observations, as well as the involvement of catecholamines in the stress response of niphargids were later confirmed by our temperature stress study (see *Publication IV*). The application of a sudden, short-term temperature elevation resulted in a change in catecholamine levels in both amphipod species, demonstrating that catecholamines are indeed involved in the physiological stress response of these organisms. In *G. pulex*, a temperature elevation from 12 to 24 °C caused a significant rise in whole-tissue NA levels. Moreover, the average DA:NA-ratio decreased with increasing temperature elevation, while the NA:A-ratio increased, indicating that the observed rise in NA levels was probably due to a conversion of DA into NA. In *N. inopinatus*, both temperature treatments (*i.e.* an elevation from 12 to 18, and from 12 to 24 °C) resulted in the occurrence of adrenaline, whereas this compound was not detected in any of the specimens in the control treatment (without temperature elevation). Due to the absence of A in the controls and the resulting variance of zero, this result could not be reliably tested with respect to statistics. Nevertheless, while the average A concentration increased with increasing temperature, the NA concentrations decreased. This indicated that during the heat stress exposure, a conversion of NA into A had probably taken place in *N. inopinatus* (see discussion section in *Publication IV*). In catecholamine biosynthesis, a sequence of chemical reactions is followed, in which DA is the precursor for NA, and NA is the precursor for A. Thus, after the same time period of heat exposure, in gammarids the former step was taking place, while in the niphargids, already the latter step occurred. It therefore seems conceivable that in niphargids the catecholamine conversion steps may be performed faster, which might be explained through a bigger storage, and consequently – a better availability – of DA in the niphargids.

With respect to *Hypothesis III*, and as a result of the adaptations of the two species to the different temperature regimes in their habitats, we had expected that *N. inopinatus* would be more sensitive than *G. pulex* towards short-term heat stress. Hence, we had predicted a pronounced difference in the catecholamine levels of the temperature-stressed versus the non-stressed niphargids, whereas the difference in gammarids should have been smaller, or even negligible. These

expectations were not met by the results of our study. Both species showed a pronounced response of catecholamine levels to the experienced short-term temperature stress. However, the observed catecholamine patterns were different indicating that the temperature stress response of the two species indeed differs. Together with the observed considerable interspecific differences in whole-tissue catecholamine levels, our results show that the physiological differences between the compared groundwater and the surface water amphipod might be much bigger than has been previously assumed.

Without doubt, further studies are needed in order to develop a sound, mechanistic explanation for the observed interspecific differences in catecholamine levels between the two species and also, in order to gain further insights into the processes underlying their physiological stress responses. Based on this knowledge, it would be furthermore worth to explore how other types of stressors, particularly toxic stress, as well as combinations of different stressors, affect the catecholamine levels in groundwater fauna. The investigation of such sublethal, individual-based effects of stress would represent a meaningful complement to the assessment of lethal effects by allowing the detection of stress in stygobites at an early stage, *i.e.* when it occurs at low levels, before irreversible damage or even mortality of the organisms occur.

4. Conclusions

Groundwater ecosystems and their services substantially contribute to human well-being and at the same time, offer habitat to a variety of unique species. The close link between the use of a resource and its biological integrity calls for an increased consideration of groundwater organisms in sustainable aquifer management and protection strategies. This thesis could provide some examples on the effects of single contaminants such as toluene, as well as temperature elevations on individual representatives of stygofauna. However, anthropogenic impacts on groundwater ecosystems act at different scales in time and space and often, one single type of human activity results in a complex action of several stressors at the same time. In order to understand the combined and overlaying effects of the stressors acting on groundwater ecosystems, more research is urgently needed. Moreover, future studies must go beyond the ecotoxicological investigations based on single individuals, but rather adopt a stronger focus on communities and the effects of disturbance to ecosystem functions and services.

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A new bioassay for the ecotoxicological testing of VOCs on groundwater invertebrates and the effects of toluene on *Niphargus inopinatus*

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ABSTRACT

A protocol was developed for testing the ecotoxicological effects of volatile organic compounds (VOCs) on groundwater invertebrates. Test substance volatility was addressed in a "closed from start to analysis"-design. Since manifestation of toxic effects may be delayed in 'slower metabolizing' organisms such as groundwater fauna, a time-independent (TI-) approach was adopted. Toluene was used as a model substance and its toxicity to the groundwater amphipod *Niphargus inopinatus* was assessed as an example. The method evaluation process considered various methodological issues such as partitioning of the toxicant between the water and the gas phase (Henry equilibrium), the possible depletion of oxygen in closed test vials, as well as microbial biodegradation of the test substance. For *N. inopinatus*, an $LC_{50,14\ days}$ of 46.6 mg L^{-1} toluene was obtained. The ultimate LC_{50} value was estimated at 23.3 mg L^{-1} toluene. No oxygen depletion occurred in the test vials and Henry equilibrium was found to be established after 6 h. The new test system proposed now awaits broad practical application.

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1. Introduction

Half of the world's drinking water derives from groundwater (IAEA, 2006). It is therefore unambiguous that groundwater systems deliver important services and goods and thus need protective measures (Danielopol et al., 2003). Moreover, apart from this rather resource-oriented view, aquifers are fascinating ecosystems. As such they harbour a great variety of unique organisms that are specifically adapted to the dark and mostly energy-limited subsurface habitats, and include "living fossils" as well as many cryptic and endemic species. In order to develop effective protection and resource management strategies, more knowledge on the reactions of the ecosystem players to disturbance, and in particular contaminants, is needed. An important tool for assessing the tolerance of groundwater fauna to anthropogenic contaminants is ecotoxicological testing. However, standard ecotoxicological bioassays as they are routinely used for surface water organisms do not seem to be appropriate for groundwater species. A very important issue arises from the specific physiological traits that groundwater organisms (stygobites) have evolved in adaptation to their habitat, striving to reduce energy expenditure in an energy-limited environment. In particular, their metabolic rates are significantly lower than those of phylogenetically related surface water species (Wilhelm et al., 2006). Such differences in metabolism can lead to a delayed manifestation of toxic effects and consequently to an overestimation of tolerance if routine toxicity tests with too short exposures (*e.g.* the 48 h *Daphnia* sp. acute immobilization test (OECD, 2004, test No. 202)) are applied. Therefore, for an adequate testing of groundwater organisms a longer test duration (*e.g.* as applied by Schäfers et al., 2001) seems a reasonable complement and will have to prove itself in application.

Apart from the aspects related to the specific metabolic characteristics of stygobites, the contaminants that are of relevance for surface waters are not necessarily the ones that are most important for groundwater. Accordingly, standard testing procedures have not been developed to suit some of the groundwater priority contaminants. In fact, volatile organic compounds (VOCs) from mineral oil (including BTEX, *i.e.* benzene, toluene, ethylbenzene, *o*-, *p*- and *m*-xylene) and chlorinated hydrocarbons belong to the most frequently found contaminants in European groundwater (EEA, European Environment Agency, 2007). Due to their characteristic physical and (bio)chemical features such as volatility, limited solubility in water, biodegradability, as well as sorption and bioaccumulation potential, these substances 'present difficulties in the execution and interpretation of standard aquatic toxicity tests' (Carpanini, 1996). Referred to as 'difficult-to-test' substances in ecotoxicological literature (OECD, 2000; Rufli et al., 1998), they require consideration of several methodological aspects in planning and designing an ecotoxicological test. Unfortunately, this has not always been taken into account in previous studies, making

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direct comparison of presently available toxicity data on VOCs to them rather complicated. For example, results have been difficult to interpret due to problems with volatility and sorption behaviour of benzene, trichloroethylene, toluene and other VOCs (as reported by Geyer et al., 1985; Snell et al., 1991). Moreover, little attention has been devoted to aspects like the distribution of volatile compounds between the water and the gas phase (Henry equilibrium), or the verification of the start- and end-concentrations in tests by analytical measurements (Ferrando and Andreu-Moliner, 1992; LeBlanc, 1980). Regarding groundwater ecotoxicology, the above-mentioned need for longer duration of tests with groundwater species, inevitably leads to an increase in the "difficulties" with respect to testing of VOCs. Additionally, only few tests have been conducted using true groundwater fauna (as pointed out by Hose, 2005) and even fewer considered the potentially slower metabolism of these animals. Instead, surface water organisms have often been used as surrogates for testing the toxicity of contaminated groundwater (*e.g.* Baun et al., 1999; Crevecoeur et al., 2011; Gersberg et al., 1995; Gustavson et al., 2000). In conclusion, to our knowledge, there is currently no study available which takes into account both the difficulties with volatile, easily degradable organic substances and the possibly delayed manifestation of toxic effects in stygofauna.

The purpose of this study was to develop a suitable test design with prolonged exposure for the ecotoxicological testing of VOCs on groundwater invertebrates. Toluene was used as a model volatile organic compound and its toxicity to the stygobitic amphipod *Niphargus inopinatus* was assessed as an example. Additionally, a set of experiments was conducted to examine the various methodological aspects related to 'difficult-to-test substances'. We tested: (i) the time required for Henry equilibrium to establish in the test vials, *i.e.* the time required in order to achieve a stable concentration of the contaminant; (ii) a possible oxygen depletion in the closed test vessels due to the respiration activity of the test organisms and due to degradation of the model substance by microbes inadvertently introduced together with the fauna, and (iii) whether antibiotics inhibit the microbial biodegradation.

2. Material and methods

2.1. Analysis of toluene concentrations

Toluene concentrations were analyzed *via* headspace GC/MS analysis directly in the vials that were used in the experiments. The only exception was the experiment concerning Henry equilibrium (described below), where the scientific question of interest required a determination of toluene levels directly in the aqueous phase. Measurements were performed on a Trace DSQ GC/MS (Thermo Electron, Germany) equipped with a Combi PAL autosampler (CTC Analytics, Switzerland), following the protocol given in Anneser et al. (2008). Ethylbenzene was used as internal standard and was added to each sample at a final concentration of 2.3 mg L^{-1} shortly before analysis. The vials were shaken for 17 min at a temperature of 70 °C to allow the volatile compounds to preferentially partition to the headspace. Subsequently, a 250 μL headspace sample was injected to a DB5-MS capillary column (Agilent Technologies, Germany) at a split ratio of 1:10. The mass spectrometer was operated in selected ion mode (SIM), targeting the ion masses 91, 92 and 106. For quantification of toluene, standards with different toluene concentrations were prepared, maintaining the same ratio of headspace to water volume and the same amount of internal standard as in the samples. From the obtained total toluene amounts, aqueous concentrations at a temperature of 10 °C were calculated according to Henry's law. For the calculation, the Henry's constant for toluene at a temperature of 10 °C ($H = 0.1295$) reported by Görgenyi et al. (2002) was used.

In the experiment investigating the time course of establishment of Henry equilibrium, toluene concentrations were specifically determined in the water compartment of the vials. For this purpose, liquid phase extraction with cyclohexane (described in detail below) was applied. Subsequently, a subsample of 1 μL of the cyclohexane extract was injected into the column of the GC/MS (column specifications, split ratio and ion masses as stated above). The oven programme started with an initial temperature of 40 °C, which was maintained for 1 min and then increased to 120 °C at a rate of 15 °C min^{-1}. Following this, the temperature was increased at a rate of 100 °C min^{-1} to reach its maximum at 300 °C, which was held for 1 min.

2.2. Ecotoxicological testing with N. inopinatus

The ecotoxicological effects of toluene on the groundwater amphipod *N. inopinatus* were investigated by means of a specifically designed test assay. The experimental setup was thoroughly evaluated in a series of methodological experiments. The test was conducted two times, with test 2 being a replicate of test 1 (except for small improvements in test procedures).

To take into account the possibly delayed toxic effects in groundwater fauna, a time-independent test design was chosen, *i.e.* an acute toxicity test with no predetermined temporal end point which continues until the toxic response has ceased or other (practical) considerations dictate that the test be terminated (Rand, 1995). The mortality data from such a time-independent (TI-) test are used to estimate an "ultimate LC_{50}". As expressed by Sprague (1969), the ultimate LC_{50} represents 'that level of the toxicant beyond which 50% of the population cannot live for an indefinite time', or in other words, the 'concentration which would kill the average [niphargid] on long exposure'. Consequently, the tests in this study were allowed to continue until mortality ceased (or nearly ceased), the only constraint to their duration being the stability of the contaminant concentration (*i.e.* test 1 was ended when no changes in mortality occurred for 13 consecutive days (on day 34), but test 2 was terminated after 23 days, when microbial biodegradation was assumed to have strongly reduced toluene concentrations in all treatments, even though mortality did not seem to have ceased by that time).

Due to the naturally low abundance of the test organisms in the field, only small numbers of animals could be used for each assay at a time (resulting in $n < 10$ for each toxicant level of the test). Therefore, in order to verify the reproducibility of the results and to examine the variability of the response to the test substance in the population, test 1 was repeated. The toluene concentrations applied in both tests are summarized in Table 1. The number of specimens tested was six per toluene concentration in test 1, and seven in test 2.

As groundwater animals are thigmotactic and have been observed to show increased movement activity when substrate is missing, 1 g of sterile quartz sand (0.5–1 mm) was added to each vial. Groundwater from the aquifer, in which test animals were caught, served as dilution water. It was filtered through a 0.22 μm membrane filter (Millex® GP, Millipore) to remove the main portion of particles and microorganisms. Prior to transferring the amphipods into the test vials, each vial was filled with the necessary amount of dilution water and cooled down to 10 °C. To avoid cannibalism (previously observed in our laboratory), only one animal was placed into each vial. The animals were allowed to acclimatize in the test vials overnight before toluene was added. During animal transfer to the test vials, an average amount of 370 μl non-sterile groundwater was introduced into each vial along with the animal (as was experimentally determined). A preliminary experiment (described below) had shown that bacteria from this groundwater started degrading the toluene in the test vials

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Table 1
Toluene concentrations [mg L^{-1}] in the two ecotoxicological tests (aqueous compartment at Henry equilibrium).

Test 1						
Nominal	2.5	6.3	15.8	24.8	39.5	63.0
Measured*	4.4/4.4	8.7/9.6	20.5/20.3	31.1/30.2	45.3/44.1	55.2/42.9
(Mean)	(4.4)	(9.1)	(20.4)	(30.6)	(44.7)	(49.1)
Test 2						
Nominal	–	9.9	19.8	39.6	80.9	159.6
Measured*	–	9.2/8.7	15.6/16.9	36.2/36.4	59.8/59.9	113.7/123.6
(Mean)	–	(9.0)	(16.3)	(36.3)	(59.8)	(118.7)

* To assess start conditions, toluene concentrations were measured on day 0 in two replicate vials that were not included in the test.

after a certain lag-phase, the latter being positively correlated with toluene concentrations. Therefore, some improvements were applied to test 2 in order to delay the start of bacterial toluene degradation, *i.e.* higher concentrations of toluene were used, and each animal was transferred to a freshly prepared rinsing vial with sterile groundwater before being introduced into the test vial. Moreover, a set of 12 additional replicate vials of the lowest toluene treatment in test 2 (including test animals) was set up for monitoring the toxicant concentration. At six different time points, two of these vials were sacrificed for toluene analysis.

As proposed by Moesslacher (2000), all tests were conducted in complete darkness, at a temperature of 10 °C, without aeration (to avoid stress to the organisms), and without the addition of food. The bioassays were performed in crimp-top glass vials for GC/MS headspace analysis (10 mL nominal volume; VWR International, Germany). This way, at the end of the test, toluene concentrations could be measured directly in the test vials, avoiding volatilization losses due to sampling of the water and/or animal transfers. To assess true start conditions in the bioassays, at day 0, toluene concentrations were additionally measured in a set of parallel vials that were not included in the test. The tightness of the test vials was verified in a separate experiment, mimicking closely the conditions of the toxicity tests. The toluene concentration in the test vials remained constant even after a continuous use for at least 5 weeks (data not shown).

Toluene (Riedel-de Haën, Germany) stock solutions were prepared in 0.22 μm-filtered groundwater in a glass bottle without headspace and sealed with a gas tight Teflon valve (Mininert™: Supelco, Germany) as follows: 2.2 mM = 206 mg L^{-1} for test 1, and 4.5 mM = 410.1 mg L^{-1} for test 2. The stock solutions were stirred for at least 24 h to allow complete toluene dissolution and subsequently cooled down to 10 °C before usage. A final volume of 3.5 mL liquid was reached in each vial after toluene addition, leaving an average of 8.2 mL headspace. Each vial was closed immediately after toluene addition with a gastight polytetrafluoroethylene (PTFE) lined cap. The amount of stock solution needed to adjust the desired nominal concentration in the aqueous compartment of the vials at a temperature of 10 °C was calculated according to Henry's law. Consequently, at the beginning of the exposure, before Henry equilibrium was established, the initial aqueous concentration of toluene in each treatment was 1.3 times higher than the values reported in Table 1. To take this into account, an additional check for mortality effects was performed 2 h after toluene addition.

Mortality in each test vial was recorded daily during the first 10 days, and later on observations were continued with maximal gaps of 3 days. 'Death' was defined as complete immobility of the animal without response of either gills, legs or antennae to slight agitation of the vial over 1–2 min of observation. Once an animal was found dead, the whole test vial was frozen until toluene analysis.

Analysis of mortality data was performed in the software environment for statistical computing R (R Development Core Team, 2008). Dose–response modelling and calculation of LC_{50} values were done using the '*drc*' package by Ritz and Streibig (Ritz, 2010; Ritz and Streibig, 2005). In detail, a 2-parameter log-logistic model (*i.e.* with the lower and upper limits fixed at 0 and 1, respectively) was used to describe mortality as a function of toluene concentration:

$$y = f(x; a, b) = \frac{1}{1 + \exp(a * (\ln(x) - \ln(b)))} = \frac{1}{1 + (x/b)^a} \quad (1)$$

with 'y' being the portion of lethally affected specimens at a certain toxicant concentration 'x'. The parameter 'b' represents the toxicant concentration that leads to 50% mortality (*i.e.* the LC_{50}), and 'a' denotes the relative slope around 'b'. The LC_{50} values were calculated based on the measured start concentrations of toluene, rather than the nominal concentrations, and toxicant concentrations were not logarithmically transformed prior to curve-fitting. For each day of observation, a separate LC_{50} was computed.

To estimate ultimate LC_{50}, the mortality data from both tests 1 and 2 were used. As the two tests had resulted in similar LC_{50} values, the mortality responses from observation days common to both tests were pooled, in order to enhance the statistical robustness of the dataset. The obtained dataset consisted of the toxicant treatments with 4.4/9.0/9.1/16.3/20.4/30.6/36.3/44.7/49.1/59.8/118.7 mg L^{-1} toluene, as well as the two controls without toluene, and the corresponding mortality data from 15 days of observation, covering a total time span of 23 days. For each day of observation, a new LC_{50} value was calculated. In order to estimate the ultimate LC_{50}, the daily LC_{50}s were plotted *versus* time and a nonlinear regression was fitted by non-linear least squares and a Gauss-Newton algorithm using the '*nls*' package in R (Bates and DebRoy, 1999). According to Baas et al. (2010), the most popular explanation for the observed decrease in LC_{50} values is, that the LC_{50} time curve directly reflects the build up of the internal concentration in time. The underlying assumption is that the test organism dies when its internal concentration exceeds a certain (individually varying) threshold. The LC_{50} would then show an exponential decay in time with a rate constant that equals the elimination rate constant of the chemical from the body. Hence, under the assumption that toluene-uptake followed a one-compartment uptake-elimination model, the time course of the LC_{50} values was modelled according to the relationship

$$y = f(t, a, b) = \frac{a}{1 - \exp(-b * t)} \quad (2)$$

where 'y' is the LC_{50} as a function of time ('t'), 'a' is the ultimate LC_{50}, and 'b' is the kinetic rate constant of elimination. However, this model resulted in a very poor description of the data, comprising an R^2-value of 0.3 and yielding an estimate of ultimate LC_{50} that was higher than the actual LC_{50} values that were observed at the end of the bioassays. Therefore, in order to obtain an ultimate LC_{50} that better corresponds to the observed time-course of LC_{50} values in our dataset, an additional (empiric) equation was also fitted to the data, using a simple exponential decay function:

$$y = f(t; a, b, c) = c + a * \exp(b * t) \quad (3)$$

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where '*c*' is the horizontal asymptote corresponding to the ultimate LC_{50}, '*a*' is the intercept, '*b*' is the decay rate of the function, and '*y*' is the LC_{50} value at observation time point '*t*'.

2.3. Test organisms and culture conditions

Test animals (*N. inopinatus*) were collected from groundwater monitoring wells on the campus of the Helmholtz Research Center in Munich (Germany). All wells are situated in a shallow Quaternary porous aquifer. A phreatobiological net-sampler (mesh size: 74 μm; modified after Cvetkov, 1968) was used to collect the amphipods from the bottom of the well, and transportation to the lab in a cooling box followed immediately. Each animal was then transferred into a separate well of a 6-well plate filled with ambient groundwater, as well as a small amount of sediment and detritus obtained together with the animals during sampling. The amphipods were acclimated to laboratory conditions in the dark, at a temperature of 10 °C for at least 1 week.

Repeated sampling of the wells had shown that the amphipod community was strongly dominated by *N. inopinatus*, with the occasional occurrence of single individuals of *Niphargus bajuvaricus*. As species determination requires microscopic examination, the affiliation of test animals to *N. inopinatus* could only be verified after the end of the test. Thus, two animals in test 1 had to be excluded from mortality data analysis because they were *a posteriori* identified as *N. bajuvaricus*.

As *N. inopinatus* is reproducing very slowly and has so far not been successfully cultured under laboratory conditions, it was not possible to obtain standardized test animals of equal age for the tests. However, efforts were made to use only animals of similar size in the experiments. The average body length of the niphargids in test 1 and test 2 was 5.1 mm (SE = 0.12), and 4.8 mm (SE = 0.04), respectively. Animals in this size range were considered adults as only rarely individuals of larger size were caught.

2.4. Inhibition of the microbial biodegradation of toluene via antibiotics

The potency of the antibiotic gentamicin to inhibit microbial toluene degradation under the conditions of our ecotoxicological tests was examined in a separate experiment for two initial toluene concentrations (10 and 30 mg L^{-1}). One half of the vials in each toluene treatment was supplemented with gentamicin (PAA Laboratories, Austria) to reach a final concentration of 50 mg L^{-1}, while the other half did not contain any antibiotics. A set of 44 vials was prepared for each toluene concentration according to the procedures in the bioassay, except that no animals were added. However, in order to mimic the conditions of the tests precisely, 370 μl of non-sterile groundwater were also introduced into each vial. At different time points (days 0/1/2/7/8/11/14/18/21/29/35), two replicate vials from each treatment were sacrificed for toluene analysis.

2.5. Check for oxygen depletion

Bacterial degradation of the test substance could have led to the depletion of oxygen in the test vials. This effect might have been even exacerbated by the respiration of the test animals, given that the procedures did not involve renewal of the test solutions. To test whether oxygen depletion was likely to occur under the conditions of our bioassay, a set of 16 replicate vials containing a sublethal amount of toluene (24 mg L^{-1}) was prepared. In six of these vials, a test animal was introduced (inevitably also leading to the introduction of approx. 370 μL of non-sterile groundwater as mentioned above). Another six vials contained no animals but were also supplemented with 370 μL of non-sterile groundwater. The remaining four vials were prepared using sterile groundwater only (negative control, no biodegradation). For measurement of oxygen concentrations, a round spot of oxygen sensitive foil (Ø 5 mm; PreSens, Germany) was glued into each vial, at a position where it would be completely covered by the test solution. These oxygen sensor spots allowed a non-invasive measurement of oxygen levels from the outside by means of optode technology. The vials were randomly arranged in an especially manufactured rack that allowed daily oxygen measurements without agitation of the vials. Only those of the vials containing an animal were carefully taken out of the rack each day in order to check for mortality. To detect the onset of microbial degradation, the concentration of toluene was monitored. For this purpose, an extra set of 10 replicate vials containing 370 μL of non-sterile groundwater was prepared. At each of five time points (day 0/7/13/16 and 22) a pair of these vials was used for analysis of toluene concentrations.

2.6. Henry equilibrium

The time until Henry equilibrium is established, and hence, the question how long the animals would be exposed to higher than nominal toxicant concentrations, was investigated for three concentrations of toluene (0.1, 1 and 30 mg L^{-1}) at a temperature of 10 °C. For each concentration, 12 replicate vials were prepared following the procedures of the bioassay, except that no animals were added. The amount of toluene present in the water at a temperature of 10 °C was determined at six time points (1, 2, 4, 6, 24 and 48 h after toluene addition) and in replicate, thus sacrificing two of the 12 vials per concentration each time. A subsample of 1 mL was taken from the water compartment and immediately covered with 0.5 mL cyclohexane (Riedel-de Haën, Germany). The samples were then vigorously shaken for 2 h for liquid-phase extraction of toluene and left undisturbed for an additional hour to allow phase separation. Finally, 100 μL of the cyclohexane phase were transferred into a 2-mL GC vial with a 200-μL glass inlay. For the analysis, 1 μL of this cyclohexane phase was injected into the GC/MS.

3. Results

In the experimental setup of our ecotoxicological study, with a liquid fraction of 3.5 mL, 1 g of sand, a headspace of 8.2 mL, and a temperature of 10 °C, the toluene concentrations in the water became stable after 6 h (indicating establishment of Henry equilibrium), regardless of the initial toluene levels (Fig. 1).

Regarding oxygen supply, no oxygen depletion could be observed in the experiment simulating the conditions of our bioassay (as demonstrated in the vials with test animal, Fig. 2). This

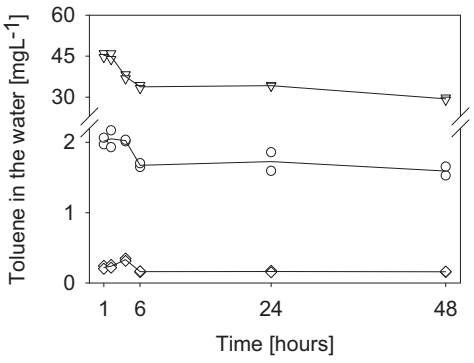

Fig. 1. Establishment of the Henry equilibrium in the test vials at a temperature of 10 °C. Toluene treatments: [◊] 0.1 mg L^{-1}; [○] 1 mg L^{-1}; [▽] 30 mg L^{-1}. Symbols show the toluene levels in each of the two replicate vials analysed per time point. Solid lines depict the time course of the respective mean values.

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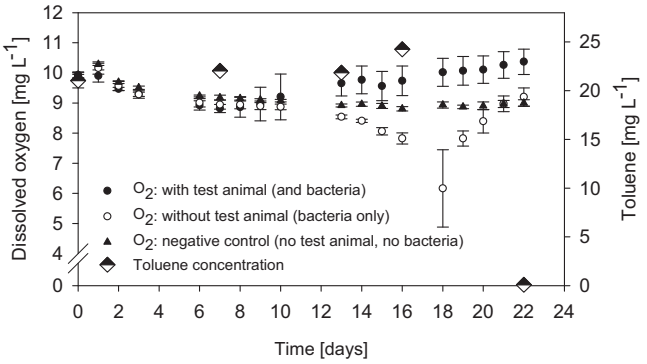

Fig. 2. Effects of toluene biodegradation and animal respiration on oxygen concentrations in the test vials. Error bars show the standard deviation of oxygen levels in each treatment. Note the differing scales for oxygen and toluene concentrations.

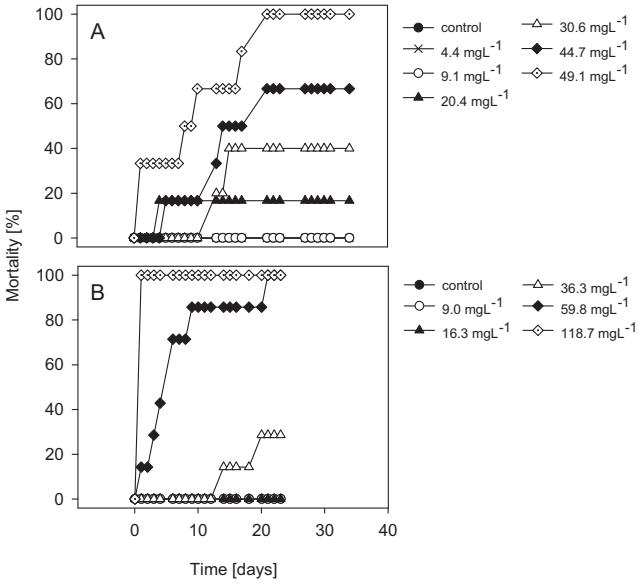

Fig. 3. Cumulative mortality data plotted against time. A: test 1; B: test 2. The symbols show the toluene treatments in terms of concentrations measured at the beginning of each test (average of two additional replicate vials per concentration). Data that are mentioned in the legend but cannot be seen in the graphs (especially controls and other treatments with 0% mortality) are being overlaid by other data points.

was the case even during the time when toluene biodegradation was taking place (shown by the decreasing toluene concentration after day 16). Likewise, no decrease in oxygen concentrations was observed in the negative controls, where no biodegradation occurred. In the test vials without amphipods, the biodegradation of toluene led to a simultaneous drop-down in oxygen. Without the locomotory activity of the animals and the slight agitations of the vial during the mortality check, diffusion alone was not fast enough to simultaneously replenish all the oxygen from the headspace. Later, as toluene mineralization came to an end, the oxygen concentration also returned to its initial level.

Toluene levels in both bioassays 1 and 2 were strongly affected towards the end by microbial biodegradation. From the onset of biodegradation, a continuous decrease in toluene concentrations occurred. Measurable biodegradation started first in the lowest toxicant levels (as revealed by the experiment with antibiotics described below). Thus, the toluene analyses on the last day of each test showed complete toxicant depletion in the low-dosed treatments and reduced toluene levels in the highly dosed treatments (data not shown). The monitoring of toluene concentrations by means of parallel vials in the lowest toluene treatment of test 2 revealed that microbial biodegradation had started to influence the toxicant levels after 14 days. For test 1, evidence from preliminary experiments (not published) indicated that measurable degradation of toluene must have begun earlier, *i.e.* around day 8. Therefore, all estimates of toxicity are given with respect to these time periods. The latest LC_{50} values that could be obtained under stable toluene concentrations were 65.4 mg L^{-1} after 8 days in test 1, and 46.6 mg L^{-1} after 14 days in test 2 (see Table 2). The corresponding NOLC values (no observed lethal concentration) were 9.1 mg L^{-1} toluene in test 1, and 16.3 mg L^{-1} in test 2. The LOLC values (lowest observed lethal concentration) were 20.4 mg L^{-1} and 36.3 mg L^{-1} respectively (Fig. 3). At a toluene concentration of 118.7 mg L^{-1} 100% mortality within 24 h was observed (test 2). No mortality occurred in the control treatments of any of the tests. Likewise, no mortality occurred as an immediate response to the initially elevated toluene concentrations as recorded during the additional mortality check 2 h after toluene addition.

The time-course of LC_{50} values of test 2 overlapped with the 95% confidence interval of the time course of test 1, indicating good reproducibility. Using Eq. (2) and the pooled data from both bioassays, an ultimate LC_{50} value of 47.8 mg L^{-1} toluene (with a 95%-confidence interval ranging from 41.4 to 54.2) was estimated. The kinetic rate constant of elimination equalled 0.98. However, the fit strongly underestimated the time-course of LC_{50}-values in the beginning (during the first week), and overestimated it towards the end. Accordingly, the R^2-value was very low and equalled 0.3. The estimated ultimate LC_{50} exceeded the observed LC_{50} values at the end of each of the two bioassays. A better (though empiric) description of the data was obtained using Eq. (3), which resulted in an R^2 of 0.99. The ultimate LC_{50} equalled 23.3 mg L^{-1} toluene (with a 95%-confidence interval ranging from 12.8 to 29.3). The parameters '*a*' and '*b*' corresponded to 49.2 and −0.06, respectively.

Table 2
Toxicity of toluene on *Niphargus inopinatus*. The LC_{50} values [mg L^{-1}] are based on the average of the initial toluene concentrations measured in the tests on day 0. On days marked with an asterisk, toluene levels in the vials were presumably lower than the nominal levels due to bacterial degradation. The abbreviation SE denotes the standard errors of the respective LC_{50} values.

Test 1				Test 2				Pooled data			
Days	LC_{50}	SE	Slope	Days	LC_{50}	SE	Slope	Days	LC_{50}	SE	Slope
1–3	**49.6**	1.4	−74.4	1–2	**65.6**	43.4	−19.4	1–2	**67.9**	7.9	−6.7
4	**140.9**	184.7	−1.4	3	**62.6**	21.5	−19.7	3	**63.5**	6.4	−7.4
5–7	**89.4**	64.6	−1.8	4	**60.7**	5.0	−18.6	4	**63.9**	7.9	−4.2
8–9*	**65.4**	26.5	−2.3	6–8	**57.4**	14.3	−22.7	6–7	**55.3**	5.5	−4.7
10*	**54.2**	13.6	−2.8	9–12	**55.7**	25.7	−25.0	8	**53.2**	5.1	−4.7
13*	**46.5**	9.6	−2.6	14–18*	**46.6**	4.9	−7.3	9	**50.8**	4.2	−5.3
14*	**42.2**	7.1	−2.9	20*	**43.0**	5.0	−5.8	10	**49.0**	3.9	−5.5
15*–16*	**39.6**	7.0	−2.7	21*–23*	**37.8**	10.0	−23.1	14	**43.3**	3.9	−4.3
17*	**36.4**	5.5	−3.1					15–16	**41.9**	4.1	−3.8
21*–34*	**32.1**	4.0	−4.3					21–23	**35.5**	2.9	−5.3

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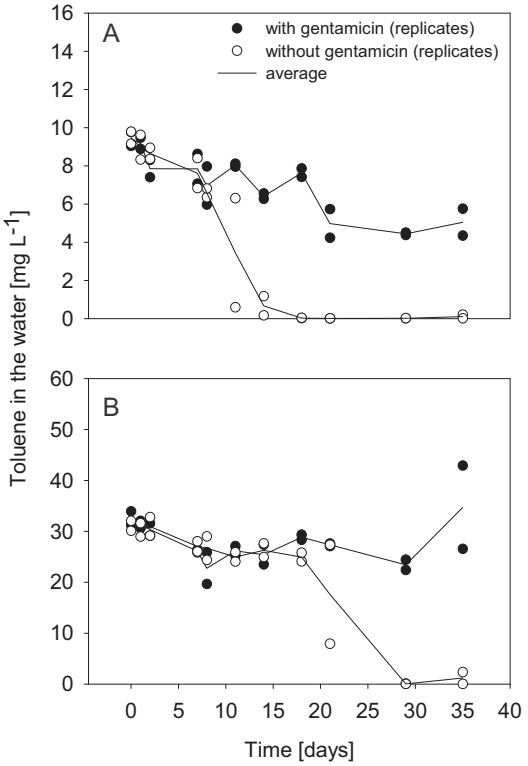

Fig. 4. Effects of the antibiotic agent gentamicin on microbial toluene degradation tested for two different initial toluene concentrations (A: 10 mg L^{-1}, and B: 30 mg L^{-1}). Solid lines represent the time course of the mean values for each of the two replicate vials.

In the experiment dealing with the inhibition of microbial biodegradation, the antibiotic agent gentamicin prevented biodegradation in both of the toluene treatments (Fig. 4). In the vials without antibiotics, the onset of measurable biodegradation was linked to the initial toluene concentration, so that the duration of the lag-phase increased with increasing toluene levels. At an initial toluene level of 10 mg L^{-1} the lag phase lasted about 8 days, and it extended to 18 days at 30 mg L^{-1} toluene.

4. Discussion

The awareness for the necessity of ecotoxicological testing with true groundwater species (stygobites) has increased during the last years, but still only a handful of studies have been performed (*e.g.* Canivet and Gibert, 2002; Krupa and Guidolin, 2003; Moesslacher, 2000; Notenboom et al., 1992). Because there are no standardized protocols available yet, these authors have chosen the durations for their tests according to the existing guidelines for surface water fauna, or based on their specific knowledge and experience. However, evidence is increasing that there are significant differences in metabolism between related surface water and groundwater species. The levels of enzymatic activity of the main key regulatory enzymes involved in the Krebs cycle and glycolysis have been found to be 1.2–8.6 times lower in hypogean than in epigean crustacean species (Hervant, 1996). Similarly, Wilhelm et al. (2006) demonstrated with a literature review, that on average the rates of oxygen consumption in stygobites are approximately two times lower than those of stygophilic or epigean species. Such pronounced differences in metabolism are believed to influence toxicant uptake rates and consequently may lead to a delayed manifestation of toxicity effects. This would in turn result in an overestimation of tolerance in the tested groundwater species if too short exposures were applied. On the other hand, the slow metabolism might also have the opposite effect by prolonging the exposure with contaminant to specific organs (due to slower depuration rates), which might then lead to a faster short-term mortality as compared to rapidly metabolizing and more flexible "relatives". Even though to our knowledge the latter case has not been documented yet, both lines of argumentation suggest that metabolism should be considered when specifying the duration of ecotoxicological studies with stygobites. Particularly, in order to create a common ground for comparison with surface water fauna, longer observations are needed–for example as in standard chronic tests with *D. magna* (21 days; OECD, 2008) or in the prolonged toxicity test for fish (14–28 days; OECD, 1984).

Compared to the acute values for other crustaceans found in literature, the LC_{50} values in this study (although obtained in prolonged acute TI-tests) were in the same order of magnitude (Table 2). For example, 7.0 mg L^{-1} toluene repeatedly killed half of the tested individuals of *Ceriodaphnia dubia* after 48 h in a study by Nunes-Halldorson et al. (2004). For *Gammarus minus*, an epigean amphipod, the US EPA Ecotox database lists an $LC_{50,\ 96\ h}$ of 58 mg L^{-1} (Horne and Oblad, 1983). Compared to the latter, the $LC_{50,\ 96\ h}$ values for *N. inopinatus* from both tests in the present study were higher, therefore indicating a lower sensitivity of the groundwater amphipod. However, as discussed above, there are serious problems with such comparisons due to the possibly differing temporal behaviour of toxicity. Moreover, as pointed out by W. F. Humphreys, hypogean species have much greater fat stores than epigean species, as well as potentially differing metabolic pathways (Humphreys, 2007). Therefore, we argue that in order to compare groundwater species and surface water species appropriately, a test with a prolonged observation of toxicity for each of the two would be needed. Until a standard procedure for groundwater species is available, a time-independent test resulting in an ultimate LC_{50} (as advised by J. B. Sprague, and more recently by Baas et al. (2010)) provides a good estimation of toxicity. Additionally, it has the advantage that ultimate LC_{50} prevents the problems arising from the comparison of different toxicity dynamics in time.

Another set of difficulties in comparing our toxicity data with the literature arises from the methodical aspects related to VOCs. As stated in the European Union risk assessment report for toluene, several studies on crustaceans (with toluene) have been performed; however, most of them have not taken volatility into account (Hansen et al., 2003). As the prolonged duration of a time-independent test even amplifies the problems associated with volatility, it was verified in advance for the present study that the test vials would remain tight until the end of the bioassay. Additionally, the toluene concentrations were measured in the beginning and at the end of the test, as well as at different time points in between. This approach allowed the detection of the onset of measurable bacterial degradation which was relevant for the interpretation of the toxicity data.

Supplementing the test vials with gentamicin successfully inhibited bacterial biodegradation of toluene in the experiment with antibiotics (Fig. 4). This result proved that the toluene losses in the bioassays should indeed be attributed to microbial biodegradation. Additionally, the experiment showed that without antibiotics, the duration of the lag-phase of microbial biodegradation increased with increasing initial toluene levels. Thus, measurable toxicant losses occurred first in the low-dosed treatments. This result is of relevance for the design of ecotoxicological studies, as it means that it is mainly important to monitor contaminant concentrations in the low-dosed treatments.

Regarding the overall temporal dynamics of LC_{50} values observed in both tests, we cannot say whether mortality had ceased according to the actual toxicity pattern in time or because of the decreasing toluene levels due to the microbial degradation of

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toluene. Hence, the lowest toluene level that is lethal for 50% of the niphargid population in the long term (*i.e.* the ultimate LC_{50}) cannot be named with certainty. However, toxicity data from those days in tests 1 and 2 when toluene concentrations were still stable (*i.e.* before microbial degradation became apparent) indicate that the ultimate LC_{50} is lower than 65.4 mg L^{-1} ($LC_{50,day\,8}$ in test 1), and also lower than 46.6 mg L^{-1} ($LC_{50,day\,14}$ in test 2). Aiming at the lowest possible (and the most conservative) critical value that can be derived from our data, a toxicity curve as a function of time was fitted despite toluene degradation in order to estimate the ultimate LC_{50}. However, the equation usually applied for this purpose (Eq. (2)) did not result in a good description of the observed time pattern. One possible reason for this might be that the toluene losses in our bioassay influenced the time course of mortality. However, this should not have influenced the results during the first week, as measurable decrease in the toluene levels of both bioassays occurred later. Therefore, this cannot account for the underestimation at the beginning of the time course. Another explanation might be the slow metabolism of stygobites, which could lead to a slower decrease in LC_{50} values as compared to the one typically observed with surface water organisms. Additionally, an interaction between these two mechanisms is also conceivable.

The ultimate LC_{50} that was obtained by fitting Eq. (3), was based on the idea that ultimate LC_{50} is reached when no further mortality occurs. Therefore, the horizontal asymptote of the LC_{50} time course corresponds to the ultimate LC_{50}. Even though fully empiric, we used this equation in order to estimate an ultimate LC_{50} that better describes the time-pattern at hand. Bearing in mind, that higher mortality might have occurred if toluene levels had remained stable till the end of the test, the resulting ultimate LC_{50} of 23.3 mg L^{-1} toluene is still probably higher than the "true" value. Nevertheless, being 64% lower than the last LC_{50} obtained with stable toluene concentrations in test 1 ($LC_{50,day\,8}$ = 65.4 mg L^{-1}), this ultimate LC_{50} value illustrates the broad range of LC_{50} variation associated with time and the resulting importance of carefully choosing the exposure duration for ecotoxicological studies with groundwater species.

For many VOCs, especially the ones that are more resistant to biodegradation than toluene, ultimate LC_{50} might be already reached before the substance starts to disappear. For all others, that are as quickly degraded as toluene, the time point of beginning concentration decline is an important factor for the interpretation of ultimate LC_{50} values. One might argue that if a substance is so easily degradable, it will probably not have a long-term impact on ecosystems. However, at sites contaminated with petroleum compounds, VOCs (for which toluene is only one model substance) are often present as part of a multicompound non-aqueous phase. As a result, contaminants are continuously released to the groundwater and stygofauna living downstream may therefore be systematically exposed to high concentrations of chemicals and experience serious toxic stress. Indeed, toluene concentrations of more than 20 mg L^{-1} have been repeatedly reported from gasworks sites (Stelzer et al., 2006; Winderl et al., 2008; Yagi et al., 2009). The toluene levels that were found to be lethal for the groundwater amphipod *N. inopinatus* in this study are therefore clearly within the range of concentrations reported from contaminated field sites, emphasizing the high environmental relevance of the results.

5. Conclusions

In this study we developed an ecotoxicological test system for groundwater invertebrates and VOCs. We propose time-independent tests (as well as other long-term tests) with true groundwater species to be more widely used in groundwater ecotoxicology in order to create an adequate basis for further developments in this field. However, testing of readily biodegradable chemicals remains difficult. In order to achieve a more realistic interpretation of the results, it is important to detect the onset of toxicant decline. As the latter is positively correlated with the initial concentration of the test chemical, monitoring of concentrations in the lowest treatment is sufficient. With toluene, the bioassay yielded robust mortality data for a duration of 8, respectively 14 days (with small method modifications). Even though oxygen depletion did not occur in this study, it cannot be excluded in closed test systems – especially if the vials are filled completely without leaving an oxic headspace. In vials with headspace, the time required to establish Henry equilibrium needs to be considered and the actual concentrations of the chemical in the water should be calculated accordingly. The resulting high initial fluctuations in toxicant concentrations should be taken into account when analysing early effects of toxicity. The experimental setup presented here can be easily adapted to any other aquatic toxicity test with volatile substances. Regarding the comparatively "young" scientific field of ecotoxicological testing with true stygobiota, we think that TI-tests represent a promising approach and should be considered where possible – at least until the necessary experience has been gained to enable a reasonable fixing of test duration for standardized test procedures.

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FACHBEITRAG

Oberflächennahe Geothermie und ihre potenziellen Auswirkungen auf Grundwasserökosysteme

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Zusammenfassung Oberflächennahe Geothermie ist eine sich rasant entwickelnde Technologie, deren Einfluss auf die Ökologie unterirdischer aquatischer Lebensräume bisher nicht ausreichend untersucht wurde. Dabei sind biologische Prozesse maßgeblich von der Temperatur beeinflusst. In Feld- und Laboruntersuchungen, die Temperaturveränderungen von 2 bis 45 °C umfassten, erwiesen sich insbesondere die Diversität und Zusammensetzung von Bakteriengemeinschaften im Aquifer als sehr temperatursensitiv, während mikrobielle Biomasse und Aktivitäten zusätzlich von der Nährstoff- und Substratverfügbarkeit im Grundwasserleiter beeinflusst waren. Echte Grundwasserinvertebraten zeigten eine geringe Temperaturtoleranz gegenüber dauerhaften Temperaturerhöhungen. Die durchgeführten Untersuchungen erlauben erste Empfehlungen für eine ökologisch nachhaltige Planung, Genehmigung, den Bau und den Betrieb von oberflächennahen Geothermieanlagen.

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Shallow geothermal energy usage and its potential impacts on groundwater ecosystems

Abstract The use of shallow geothermal energy is a thriving technology. Still, its impact on the ecology of subsurface habitats has not been adequately investigated. Biological processes are substantially influenced by temperature. In field and laboratory investigations comprising a temperature range from 2 to 45 °C we show, that the diversity and structure of aquifer microbial communities is significantly influenced by temperature. Microbial biomass and activities are shown to additionally depend on the availability of nutrients and substrates in the groundwater. Selected groundwater invertebrates exhibited little tolerance towards mid- and long-term exposure to increased temperatures. Our results allow first recommendations towards the design, authorization, construction and operation of shallow geothermal energy facilities in an ecologically sustainable way.

Einleitung

Die Nutzung thermischer Energie aus dem Untergrund gewinnt vor dem Hintergrund der Endlichkeit fossiler Ressourcen zunehmend an Bedeutung. Weit verbreitet und mit enormen Wachstumsraten verbunden, ist die direkte Nutzung oberflächennaher (< 400 m Tiefe) thermischer Ener-

gie (flache Geothermie) zu Heiz- und Kühlzwecken mittels Erdwärmesonden (EWS) oder Grundwasserwärmepumpen (GWWP) (Sanner et al. 2003). Diese Technologien sind auch bei einem normalen geothermischen Gradienten effizient und daher fast standortunabhängig einsetzbar. In den 27 Ländern der EU betrug die realisierte Gesamtkapazität im Jahr 2008 ca. 8.920 MW (Deutschland: 1.653 MW) mit insgesamt ca. 782.461 installierten Erdwärmepumpensystemen (Deutschland: 150.263) (EUROBSERVER 2009). In Deutschland mit einer durchschnittlichen CO_2-Emission für die Stromerzeugung von 594 g CO_2/kWh können durch den Einsatz von strombetriebenen Erdwärmesonden mit einer Jahresarbeitszahl von 4 mindestens 35 % der CO_2-Emissionen gegenüber konventionellen Heizsystemen eingespart werden. Eine EWS-Anlage mit einer Leistung von 11 kW führt somit zu einer Reduktion von 1,8 t CO_2 pro Jahr (Blum et al. 2010).

Die Einrichtung und der Betrieb von Anlagen der oberflächennahen Geothermie sind mit Umwelteingriffen verbunden. Hierzu zählen je nach Standort die Veränderung von Landschaft und Landnutzung, Lärm, Untergrundstörungen (durch Bohrungen) sowie Emissionen in die Atmosphäre, in Oberflächengewässer und den Untergrund, vor allem aber Veränderungen der Grundwassertemperatur, entweder durch Erwärmung oder Abkühlung (Rybach 2003, Kristmannsdottir & Armannsson 2003, Saner et al. 2010). Schwerpunkt dieses Beitrags sind die bisher wenig untersuchten Auswirkungen auf den Untergrund und auf Grundwassersysteme. Speziell diskutiert werden mögliche Risiken für die Grundwasserqualität – etwa 75 % des deutschen Trinkwassers werden aus Grundwasser gewonnen (BMU 2008) – sowie für die Ökologie der unterirdischen aquatischen Lebensräume.

In oberflächennahen und *offenen* Systemen, z. B. einer Grundwasserwärmepumpe, wird Grundwasser direkt von einem (meist) oberstromigen Förderbrunnen zum Wärmetauscher geleitet und anschließend über Injektionsbrunnen unterstromig in den Aquifer abgegeben. *Offene* Systeme sind vor allem in der Industrie zur Abführung von Prozesswärme weit verbreitet. Doch auch für die Gebäudeklimatisierung werden mancherorts *offene* Systeme genutzt. Dabei wird dem Untergrund im Winter Wärme entzogen, verbunden mit einer Abkühlung des Grundwasserleiters, während im Sommer Wärme aus der Gebäudekühlung in den Untergrund verbracht wird. Die Ausdehnung der von Temperaturveränderungen beeinflussten Bereiche hängt stark von den hydraulischen Eigenschaften des Grundwasserleiters ab (Umweltministerium Baden-Württemberg 2009, Berner et al. 2010). Die häufigste Form der oberflächennahen Geothermie aber sind *geschlossene* Systeme (z. B. Fridleifsson et al. 2008). Hier werden Rohre mit zirkulierender Sole entweder horizontal in einer Tiefe von 1–2 m (Erdwärmekollektoren) oder vertikal bis in Tiefen von 50–400 m (Erdwärmesonden) in den Untergrund verbracht. Auch hier wird die Ausdehnung der Wärmefahnen durch die hydraulische Durchlässigkeit der durchströmten Sedimente, die Grundwasserfließgeschwindigkeit sowie durch die entnommene Wärmemenge bestimmt (Pannike et al. 2006, Hähnlein et al. 2010a). Bei großen Gebäuden bedarf es einer Vielzahl von Erdwärmesonden (Sondenfeldern oder -galerien), sodass hier im Vergleich zu Einzelanlagen größere Bereiche des Untergrunds von Temperaturveränderungen betroffen sind.

Die natürlichen Temperaturschwankungen im Grundwasser und die Tiefe der ganzjährig *isothermen* Zone hängen sehr stark vom Wärmeleitvermögen des Untergrunds ab. Wird als Grenzamplitude 0,1 °C gewählt, so liegt die *isotherme* Zone in den gemäßigten Breiten bei etwa 15 m (Mattheß 1994). Im Verhältnis zur natürlichen Grundwassertemperatur bewegen sich die durch oberflächennahe Geothermie verursachten Temperaturveränderungen im Bereich ±5 K für EWS und im Bereich von ±10 K für GWWP (Hähnlein et al. 2010b). Das kann in manchen Grundwasserleitern in jahreszeitlichen Schwankungen bis unter 4 °C im Winter und bis 20 °C und darüber im Sommer resultieren. Mit der Temperaturveränderung kann es auch zu physikalischen und chemischen Veränderungen des Wassers kommen. Die Temperatur beeinflusst die Dichte und Viskosität des Wassers und damit die Fließgeschwindigkeit sowie bestimmte Lösungsgleichgewichte für Feststoffe, Flüssigkeiten und Gase (Stumm & Morgan 1995). Die Einleitung erwärmten Wassers in den Untergrund kann zu Karbonatausfällungen (Griffioen & Appelo 1993), einer erhöhten Lösung von silikatischen Mineralien (Arning et al. 2006), der Mobilisierung von organischem Material und einer vermehrten CO_2-Abgabe aus Sedimenten (Brons et al. 1991) sowie einer geringeren Sauerstoffsättigung führen (Stumm & Morgan 1995). Problematisch können ebenfalls Leckagen bei geschlossenen Anlagen sein. Wärmepumpen und das Trägermedium in EWS beinhalten für gewöhnlich Frostschutzmittel (z. B. Ethylenglykol, Propylenglykol, Betain). In EWS finden sich oft noch zusätzliche Korrosionshemmer und Biozide, welche bei Leitungsbruch ins Grundwasser gelangen (Klotzbücher et al. 2007).

Bei der Bewertung der Umweltverträglichkeit flacher geothermischer Anlagen bleibt bisher meist unberücksichtigt, dass Grundwasserleiter komplexe Ökosysteme und Lebensraum für vielfältige Organismengemeinschaften sind (Griebler & Mösslacher 2003). Mikroorganismen sind überall im Untergrund in hohen Individuendichten anwesend und maßgeblich an allen Stoffkreisläufen beteiligt (Hunkeler et al. 2006, Griebler & Lueders 2009). Mikroorganismen in Grundwasserleitern sind vor allem *psychrophil* (kälteliebend, Wachstumsoptima zwischen 10 und 20 °C) und *mesophil* (Wachstumsoptima zwischen 20 und 40 °C) (Abb. 1).

Neben den ubiquitär verbreiteten Mikroorganismen leben im Grundwasser auch eine große Zahl mehrzelliger Tiere, sogenannte Metazoen. Vertreter dieser Meiofauna leben

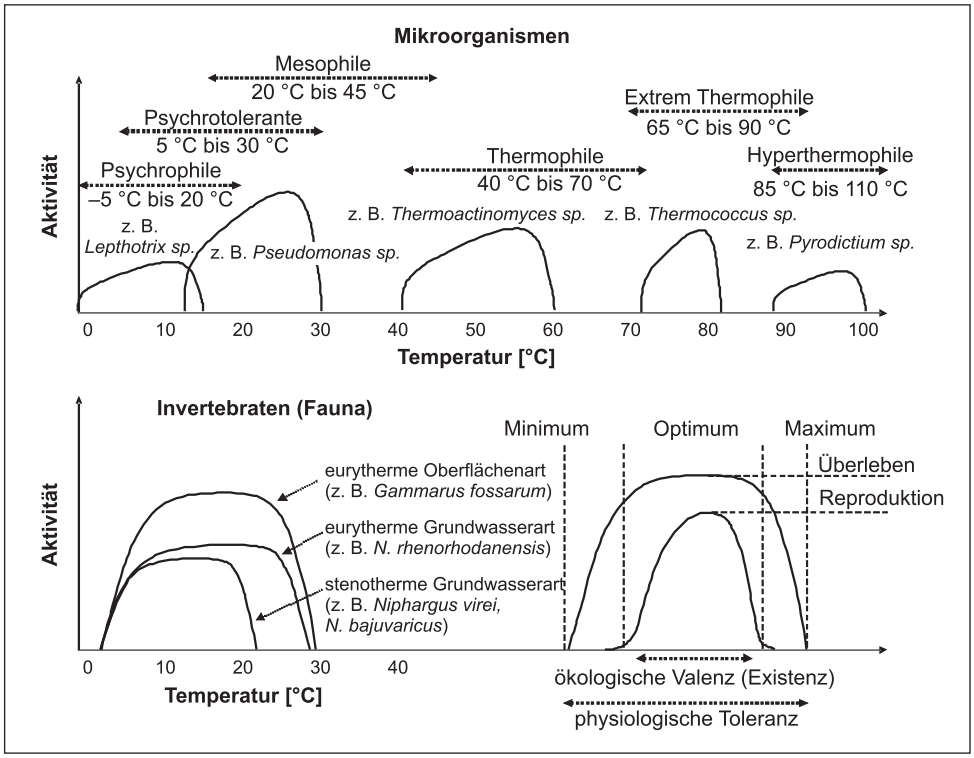

Abb. 1 Anpassung von Mikroflora und Meiofauna an verschiedene Temperaturbereiche. Jede Art hat ihren ganz spezifischen Temperaturtoleranzbereich. Die ökologische Valenz, also der Bereich, in der die jeweilige Art in der Umwelt angetroffen wird (Wachstumsoptima), ist noch wesentlich enger als ihr Toleranzbereich (Grenztemperaturen) (zusammengestellt aus Lengeler et al. 1999, Madigan et al. 2008 und Fuchs & Schlegel 2006)

vor allem oberflächennah und sind sehr heterogen (‚patchy') verteilt (Hahn & Matzke 2005). Gerade die oft sehr kohlenstoff- und nährstoffarmen (oligoalimonen) und temperaturkonstanten Lebensbedingungen haben bei den höheren Organismen über geologische Zeiträume hinweg zu erheblichen Anpassungen geführt. Echte Grundwasserarten (Stygobionten) innerhalb der Invertebraten sind in der Regel blind, pigmentlos, haben einen niedrigen Basisstoffwechsel und eine hohe Hungertoleranz (Griebler & Mösslacher 2003).

Den unterirdischen Lebensräumen wird eine Reihe essentieller ökosystemarer Dienstleistungen zugeordnet, wie z. B. Trinkwasserproduktion, Schadstoffabbau, Rückhalt von Nährstoffen oder Eliminierung von pathogenen Mikroorganismen (Herman et al. 2001, Boulton et al. 2008, Griebler & Lueders 2009, Avramov et al. 2010). Insbesondere Änderungen der Temperatur können einen großen Einfluss auf die Biologie und demzufolge auch auf diese Ökosystemdienstleistungen haben. Daher ist eine umfassende Berücksichtigung ökologischer Aspekte bei der Genehmigung von Geothermieanlagen durch Behörden notwendig.

Ziel der in diesem Artikel vorgestellten Arbeiten ist es, bisherige Erkenntnisse über die direkten und indirekten Effekte einer Temperaturveränderung in Grundwasserleitern zusammenzufassen und erste Empfehlungen für die Genehmigung, die Planung, den Bau und den Betrieb von oberflächennahen Geothermieanlagen abzuleiten. Hierzu wurden (1) eine Feldstudie (Brielmann et al. 2009) über die Auswirkungen eines offenen Systems auf die Ökologie in einem sehr sauberen und hoch durchlässigen quartären Grundwasserleiter im Raum Freising (Bayern), (2) Laborversuche an mit Standortmaterial gefüllten Sedimentsäulen und (3) Experimente zur Temperaturtoleranz von ausgewählten Grundwasserinvertebraten durchgeführt.

Material und Methoden

Feldstudie

Im Sommer 2007 wurde in Freising (Bayern) eine 1,5 km lange Temperaturfahne in einem oberflächennahen quartären Kiesgrundwasserleiter über den Zeitraum von einem Jahr untersucht. Die lokale Grundwassererwärmung ist durch ein *offenes* System verursacht. Für Kühlzwecke wird Grundwasser in großen Mengen (3.000 m^3/h) oberstromig einer Industrieanlage entnommen und unterstromig über Schluckbrunnen wieder dem Grundwasserleiter zugeführt. Die mittlere natürliche Grundwassertemperatur im Untersuchungsgebiet beträgt 11 ± 1 °C. Acht ausgewählte Messstellen umfassen die Temperaturfahne und von der Erwärmung unbeeinflusste Teile des quartären Kiesgrundwasserleiters (mittlere Tiefe 8–15 m unter Geländeoberkante). Die Messstellen wurden in ‚unbeeinflusst', ‚zeitweise beeinflusst', und ‚kontinuierlich beeinflusst' untergliedert. Untersucht wurden hydrochemische Parameter (Tempera-

tur, elektrische Leitfähigkeit, pH, Sauerstoffgehalt, Redoxpotential, Hauptionen, Orthophosphat und gelöster organischer Kohlenstoff (DOC) sowie die Abundanz, Aktivität und Diversität der Bakteriengemeinschaften und der Meiofauna. Eine detaillierte Beschreibung der Analysemethoden und der Ergebnisse finden sich in Brielmann et al. (2009). Die hohe hydraulische Leitfähigkeit ($k_f = 0{,}023\ \mathrm{m \cdot s^{-1}}$), die hohen Grundwasserabstandsgeschwindigkeiten von 18 bis 29 $\mathrm{m \cdot d^{-1}}$, sowie die teilweise Entwässerung des Grundwasserleiters in die angrenzenden Isarauen und die deshalb natürlicherweise begrenzte Ausbreitung der Temperaturfahne begünstigen die thermische Nutzung von Grundwasser an diesem Standort.

Säulenversuch

In einem Säulenexperiment mit Standortsediment (Abb. 2) wurde der Temperatureinfluss auf bakterielle Gemeinschaften im Grundwasser (suspendierte Zellen) und im Sediment (festsitzende Zellen) unter kontrollierten Bedingungen untersucht. Die Mittelsandfraktion (0,2–0,63 mm) dieser Sedimente wurde in jeweils 6 Säulenreplikaten bei Temperaturen von 4, 10, 15, 20, 30 und 45 °C inkubiert und bei Pumpraten von 0,6 $\mathrm{ml \cdot min^{-1}}$ (Abstandsgeschwindigkeit ~11 $\mathrm{m \cdot d^{-1}}$) kontinuierlich mit Grundwasser durchströmt. Nach einer Adaptationsphase von etwa vier Monaten wurde am Auslass der Säulen der pH-Wert, die Sauerstoffkonzentration, die Hauptionen, Orthophosphat und der DOC sowie die bakterielle Abundanz, Aktivität und Diversität bestimmt. Die Analyse dieser Parameter erfolgte wie in Brielmann et al. (2009) beschrieben, aber mit reduzierten Probenvolumina für die DNA-Extraktion (800–1.000 ml) und die Bestimmung der Gesamtzellzahl (10 ml). Das für die DNA-Extraktion am Säulenauslass gesammelte Wasser wurde während der gesamten Probennahmezeit auf Eis gekühlt.

Neben dem Säulenausfluss wurden auch die Säulensedimente, wie im Folgenden dargestellt, hinsichtlich der bakteriellen Abundanz, Aktivitäten und Diversität untersucht. Für die Bestimmung der Gesamtzellzahl wurden 0,75 ml Sediment mit 2,5 % iger Glutaraldehydlösung fixiert und wie in Anneser et al. (2010) beschrieben, analysiert.

Bakterielle Kohlenstoffproduktion (BKP) als Indikator für die mikrobielle Aktivität wurde über die Inkorporation von ^{3}H-markiertem Thymidin in bakterielle DNA, modifiziert nach Findlay et al. (1984), Bååth (1990) und Kirschner & Velimirov (1999) bestimmt. Jeweils 4 Replikate einer 0,5 ml Sedimentprobe wurden mit 750 µl sterilfiltriertem Grundwasser und 100 µl einer 100 nM [Methyl-^{3}H]-Thymidin-Arbeitslösung (85 Ci/mmol, 1 m $\mathrm{Ci \cdot ml^{-1}}$, GE Healthcare) für 3 Stunden bei entsprechender Versuchstemperatur inkubiert. Ein Replikat wurde als Kontrolle unmittelbar nach der [Methyl-^{3}H]-Thymidinzugabe mit Formaldehyd (5 % Endkonzentration) abgestoppt, die anderen nach Ablauf der Inkubationszeit. Die fixierten Proben wurden bis zur weiteren Bearbeitung bei 4 °C aufbewahrt. Zur Extraktion der DNA wurden die Proben bei 15.000 g für 10 Minuten zentrifugiert und der Überstand verworfen. Nach zwei Waschschritten mit jeweils 900 µl Reinstwasser (Millipore) wurden die Proben mit 900 µl einer alkalischen Lösung (0,6 M NaOH, 0,1 % SDS, 25 mM EDTA) für 1 h bei 99 °C und 1.000 U/min auf einem Thermoschüttler extrahiert, abgekühlt und erneut bei 15.000 g für 10 min zentrifugiert. Aus dem Überstand wurde ein 100 µl Aliquot mit Szintillationscocktail versetzt und in einem Flüssigszintillationszähler (Canberra Packard Tricarb 1600 TR) gemessen. Ergänzende Tests zeigten, das nur ca. 10 % des gesamten aufgenommenen [Methyl-^{3}H]-Thymidin-Labels in die bakterielle DNA inkorporiert wurden. Für die Berechnung der Kohlenstoffproduktionsraten wurden die Umrechnungsfaktoren nach Bell (1990): $1 \cdot 10^{18}$ $\mathrm{Zellen \cdot mol^{-1}}$ und Griebler et al. (2002): 20 fg C $\mathrm{Zelle^{-1}}$ verwendet.

Extrazelluläre Phosphatase-Aktivität (EPA) im Säulensediment wurde in Anlehnung an Wobus et al. (2003) bestimmt. Methylumbelliferyl-Phosphat (MUF-P, Sigma) wurde als Substrat und 4-Methylumbelliferon (MUF, Sigma) als Standard verwendet. Eine Stammlösung MUF-P mit einer Konzentration von 10 $\mathrm{mmol \cdot l^{-1}}$ wurde unter Zugabe von 3 Vol-% Methoxyethanol hergestellt und bei −20 °C aufbewahrt. Zur Bestimmung der EPA wurden 0,5 ml Sediment in 9,75 ml sterilisiertem (Grund-)Wasser verdünnt und (bis auf die Kontrollen) mit 250 µl der MUF-P-Stammlösung versetzt (250 $\mathrm{\mu mol \cdot l^{-1}}$ Endkonzentration). Die Endkonzentration lag dabei zwar unter dem Sättigungsbereich wurde aber wegen einer besseren Vergleichbarkeit der Ergebnisse zur vorangegangenen Feldstudie (Brielmann et al. 2009) gewählt. Die Proben wurden für 3 h bei der entsprechenden Versuchstemperatur inkubiert und anschließend bei 4 °C und 3.345 g (4.000 U/min) für 5 min zentrifugiert. Aus dem Überstand wurden 3 ml entnommen und mit 300 µl eines Ammonium-Glycin-Puffers (pH 10,5) versetzt. Die Fluoreszenzmessung erfolgte unmittelbar bei 363 nm (Anregung) und 446 nm (Emission) (Bowman Series 2 Spectrofluorometer). Die Quantifizierung des Fluoreszenzproduktes erfolgte durch schrittweise Zugabe der Standardstammlösung (MUF, 100 $\mathrm{\mu mol \cdot l^{-1}}$) zu den Kontrollen (Standardadditionsverfahren).

Die Sedimentproben zur Bestimmung der Struktur der bakteriellen Lebensgemeinschaften wurden unmittelbar nach Entnahme aus den Säulen bei −20 °C bis zur weiteren Bearbeitung gefroren. DNA wurde aus ~1 g Sediment extrahiert (Winderl et al. 2008) und bei −20 °C gelagert. Anschließend wurden bakterielle 16S rRNA-Gen PCR-Produkte generiert und über T-RFLP-(Terminaler Restriktionsfragment-Längen-Polymorphismus) Fingerprinting unter Verwendung der Primer Ba27f-FAM/907r sowie des

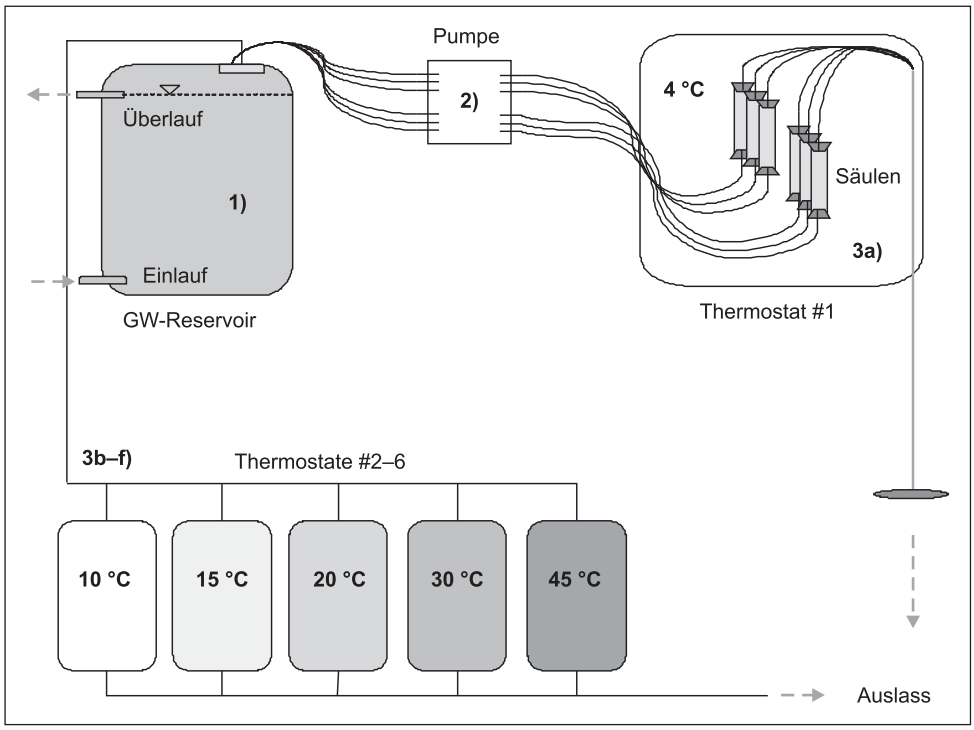

Abb. 2 Aufbau des temperaturkontrollierten Säulenversuchs. (1) Das Reservoir wurde kontinuierlich durch Grundwasser aus einem quartären Karbonatgrundwasserleiter erneuert. (2) Peristaltikpumpen hielten ein homogenes Fließsystem mit Flussraten von ∼0,6 ml/min aufrecht. Jede Pumpe strömte über Stahlkapillaren konstanter Länge insgesamt 8 Säulen ($L = 10$ cm, $\varnothing = 1{,}6$ cm) an. (3) In jedem Thermostat wurden insgesamt 6 Säulen inkubiert und von unten nach oben mit Grundwasser durchströmt. Grundwasser konnte an jeder Säule über die entsprechenden Auslässe (Stahlkapillaren) entnommen werden

Restriktionsenzyms *Msp*I analysiert, wie in Winderl et al. (2008) beschrieben.

Temperaturgradientenkammer

In einem Glaszylinder (L: 40 cm, B: 4 cm, H: 4 cm) wurde mithilfe eines Peltier-Kühlelements an einem Ende und zweier Heizfolien am anderen Ende ein Temperaturgradient (2 bis 35 °C) etabliert. Um eine stabile Temperaturschichtung zu garantieren und optimalen experimentellen Zugang zum System zu gewährleisten, wurde der Zylinder schräg gestellt (Abb. 3). Die Temperaturgradientenkammer wurde bei absoluter Dunkelheit in einer Kühlkammer installiert. Das Verhalten ausgewählter Stygobionten (Flohkrebs *Niphargus inopinatus* [Amphipoda], Assel *Proasellus cavaticus* [Isopoda]) wurde untersucht, indem je Versuch 4–5 Individuen einer Art mithilfe einer langen Pipette im Temperaturbereich von 10–12 °C abgesetzt wurden. Anschließend wurde mit einer kleinen Diodentaschenlampe die Position der Tiere alle 30 min über einen Zeitraum von 5 h protokolliert. Nach einer Beobachtungspause von etwa 12 h wurde die Position der Tiere erneut über weitere 4 h halbstündlich erfasst. Kontrollversuche wurden in derselben Kammer ohne Temperaturgradient bei einer Temperatur von 12–13 °C durchgeführt. Über den gesamten Temperaturgradienten wurde zudem mittels eines nicht-invasiven Messverfahrens (Presens Precision Sensing) Sauerstoff bestimmt, um Sauerstoffzehrung als Einflussparameter auf die Verteilung der Stygobionten auszuschließen.

Temperatur-Dosis-Wirkungs-Beziehungen

Die Temperaturtoleranz von *Niphargus inopinatus* und *Proasellus cavaticus* wurden mittels klassischer Dosis-Wirkungs-Versuche (Tox-Test) untersucht. Dazu wurden jeweils 5 bis 6 Individuen einer Art bei 6 unterschiedlichen Temperaturen (4, 8, 12, 16, 20 und 24 °C) inkubiert. Die Inkubation der Tiere erfolgte in sogenannten *Six-Well*-Platten. Jeder Behälter enthielt Grundwasser und etwas natürliches Brunnensediment. Alle Platten wurden abgedeckt, um den Wasserverlust durch Evaporation gering zu halten. Verdunstetes Wasser wurde durch Grundwasser ersetzt. Zu Versuchsbeginn wurden die Tiere kontinuierlich an die verschiedenen Temperaturen akklimatisiert. Die Temperatur-Tox-Tests wurden dynamisch ausgewertet, d. h. nach 24 h und 48 h, wie es für Tox-Tests üblich ist, und zusätzlich über einen weiteren Zeitraum von mehreren Wochen, um der verringerten Stoffwechselaktivität von Grundwasserorganismen Rechnung zu tragen. Die Ergebnisse wurden mithilfe der Bibliothek DRC 2.0-1 im Programm ‚R' (Version 2.9.0) als Dosis-Wirkungs-Diagramme ausgewertet.

Datenanalyse

Zur Abschätzung der Diversität der untersuchten Bakteriengemeinschaften wurde der Shannon-Wiener-Index H', die Shannon-Evenness E und Richness S aus den relativen Abundanzen der gemessenen bakteriellen T-RF's berechnet

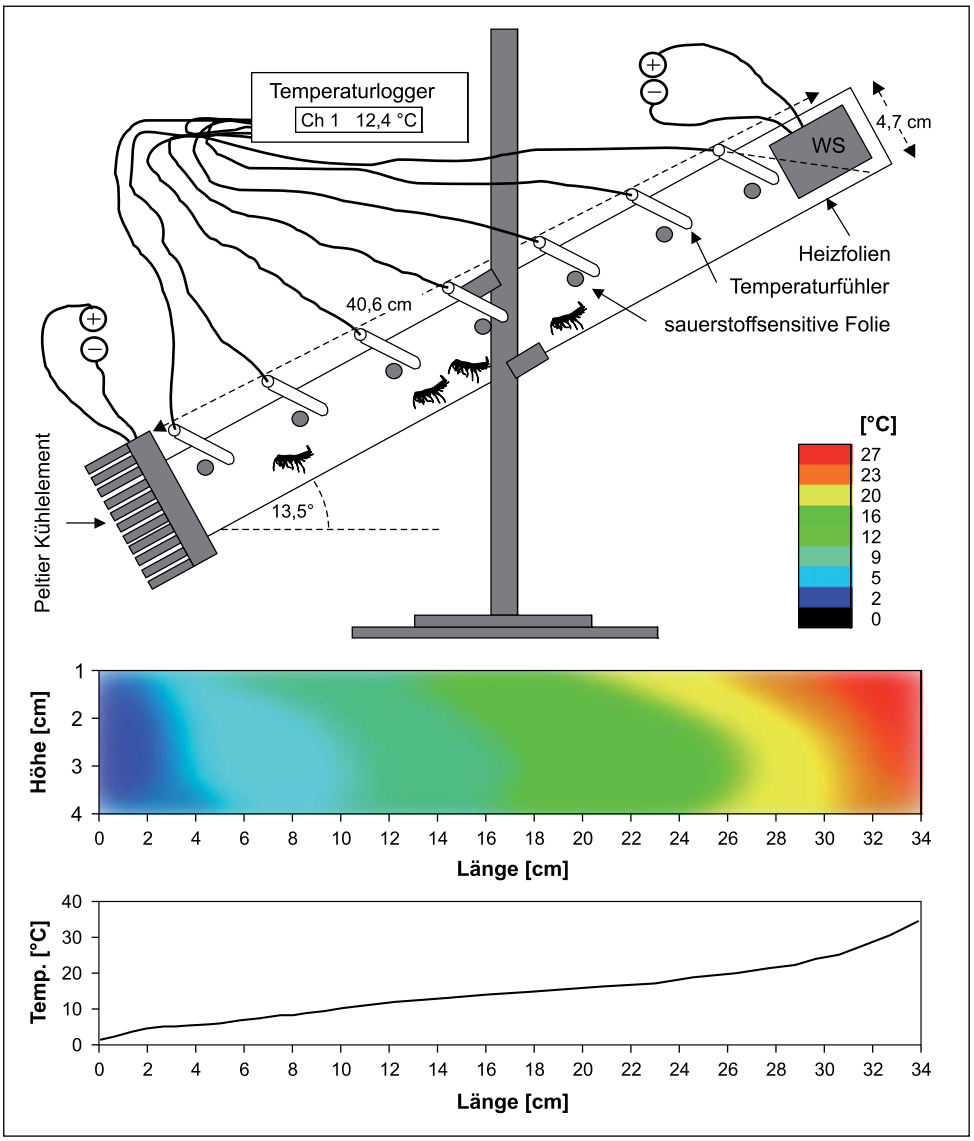

Abb. 3 Aufbau der Temperaturgradientenkammer modifiziert nach Schreglmann (2010). Der Glasquader (40,6 × 4,7 × 4,7 cm) wurde auf der einen Seite mit einem thermoelektrischem Peltier–Kühlelement mit Ventilator (3 °C) und Temperatursensor ausgestattet; auf der anderen Seite wurden Heizfolien (30 °C) angebracht. Die Kammer wurde in einem Winkel von 17,5 ° aufgestellt, um konvektiven Wärmetransport zu vermeiden. Der Boden wurde mit rauem Sandpapier ausgekleidet. Jeweils 7 Temperatursensoren und 7 Sauerstoffsensoren wurden an den Positionen 4, 9, 14, 19, 24, 29 und 34 cm in einer Höhe von 1 cm über dem Quaderboden angebracht. Die Sauerstoffmessung erfolgte mittels Lichtleiteroptodentechnik (SP-PSt3, Fibox 3, Presens Precision Sensing)

(Hill et al. 2003). Die Prüfung auf signifikante ($p < 0{,}001$) Unterschiede zwischen den Mittelwerten der hydrochemischen und mikrobiellen Parameter erfolgte mittels einseitiger ANOVA (Holm-Sidak-Test); Gefundene Abhängigkeiten wurden mittels Spearman-Rank-Korrelationen überprüft (Statistik-Paket in SigmaPlot 11.0). Der Einfluss der Temperatur auf die Zusammensetzung der Bakteriengemeinschaften im Säulenwasser und Sediment wurde mittels MANOVA wie in Brielmann et al. (2009) beschrieben und über multivariate Regressionsbäume (*multivariate regression trees* = MRTs) nach De'ath (2002) untersucht. MRT's erlauben es, die Zusammensetzung komplexer Gemeinschaften (*response variables*), insbesondere die Artenhäufigkeit, durch Umweltfaktoren (*explanatory variables*) zu erklären bzw. vorherzusagen. Mittels MRT werden in sich homogene Cluster bestimmt, die durch Umweltfaktoren definiert werden. Auf diese Weise können nicht nur Gemeinschaftstypen, sondern auch dazugehörige Habitattypen beschrieben werden. Die Analysen wurden in ‚R' (Version 2.7.0) unter Verwendung der Bibliotheken VEGAN 1.15-0 und MVPART 1.2-6 ausgeführt.

Ergebnisse

Feldstudie

Der untersuchte flache quartäre Grundwasserleiter im Münchner Norden erwies sich als sauerstoffreich und oligoalimonisch (arm an organischem Kohlenstoff und Nährstoffen) mit vergleichsweise niedrigen mittleren jährlichen Konzentrationen an DOC ($1{,}3 \pm 0{,}4$ mg $\cdot l^{-1}$), PO_4^{3-} (46 ± 23 µg $\cdot l^{-1}$ P) und NO_3^- ($15{,}0 \pm 3{,}2$ mg $\cdot l^{-1}$) (Tab. 1). Die Zufuhr großer Wärmemengen über das Kühlwasser führte zur Ausbildung einer 1,5 km langen Temperaturfahne,

Tab. 1 Physikalische und chemische Zusammensetzung der im Feld und in den Säulen untersuchten Grundwässer. Daten sind als Mittelwerte (MW) ± Standardabweichung (σ) gegeben

	pH	PO_4^{3-} [$\mu g \cdot l^{-1}$]	DOC [$mg \cdot l^{-1}$]	O_2 [$mg \cdot l^{-1}$]	Cl^- [$mg \cdot l^{-1}$]	NO_3^- [$mg \cdot l^{-1}$]	SO_4^{2-} [$mg \cdot l^{-1}$]	HCO_3^- [$mg \cdot l^{-1}$]	Na^+ [$mg \cdot l^{-1}$]	K^+ [$mg \cdot l^{-1}$]	Mg^{2+} [$mg \cdot l^{-1}$]	Ca^{2+} [$mg \cdot l^{-1}$]
Feld												
MW	7,44	35	1,0	8,48	30,79	7,6	11,33	329	16,77	1,65	19,95	84,26
σ	0,80	18	0,2	0,54	0,36	0,3	0,41	6	1,06	0,10	0,41	4,99
Säulen												
MW	7,17	46	1,3	4,00	29,78	15,0	33,30	302	19,27	3,23	20,15	82,84
σ	0,21	23	0,4	1,10	2,89	3,2	2,21	15	1,74	0,20	0,92	4,25

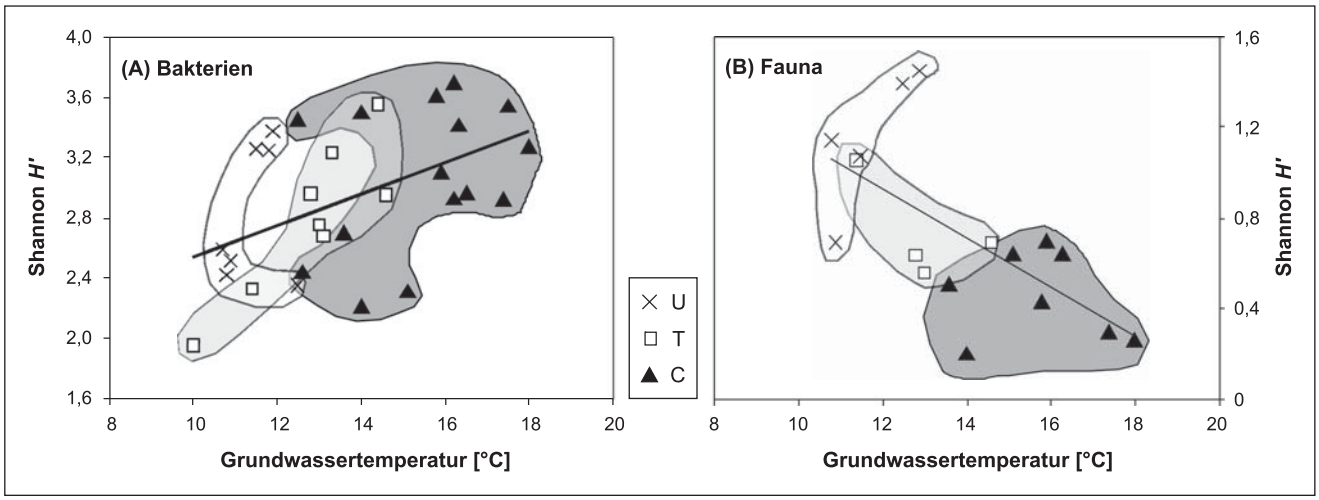

Abb. 4 Diversität nach Shannon-Wiener [H'] in Abhängigkeit von der Grundwassertemperatur für (**A**) die bakteriellen Gemeinschaften und (**B**) die Grundwasserfauna. Proben wurden zu 4 Zeitpunkten über's Jahr verteilt an ausgewählten Grundwassermessstellen entnommen; Bakterien entstammten dem gepumpten Grundwasser und die Fauna wurde im Pegelsumpf unter Verwendung eines speziellen Netzsammlers (Fuchs 2007) entnommen. U = unbeeinflusst, T = zeitweise beeinflusst, C = kontinuierlich beeinflusst (verändert nach Brielmann et al. 2009)

mit saisonal schwankender Ausbreitung und gemessenen Höchsttemperaturen von 19 °C in den Sommermonaten. Im Grundwasser des stark durchlässigen Aquifers zeigten funktionelle Parameter wie etwa die bakterielle Kohlenstoffproduktion (0,02 bis 0,81 $ng \cdot C \cdot l^{-1} \cdot h^{-1}$) keine signifikanten Veränderungen in Abhängigkeit zur Temperatur. Die Gesamtzellzahl (1,4 bis 5,4 · 10^4 $Zellen \cdot ml^{-1}$) und die Lebendkeimzahl unterlagen keinen maßgeblichen Veränderungen. Auch konnte im Freiland kein gehäuftes Auftreten von coliformen Bakterien und *E. coli* in temperaturbeeinflussten Bereichen beobachtet werden (Brielmann et al. 2009). Allein die Zusammensetzung der Lebensgemeinschaften reagierte signifikant auf die Temperaturveränderungen im Grundwasserleiter. Bereiche mit höherer Temperatur waren durch eine erhöhte Biodiversität in den Bakteriengemeinschaften charakterisiert, wohingegen ein gegenläufiger Trend für die Grundwasserfauna gefunden wurde (Abb. 4). Mit zunehmender Temperatur nahm die Artenvielfalt innerhalb der Fauna ab. Zur beobachteten saisonalen Dynamik und biologischen Gesamtvariabilität im Grundwasserleiter trugen aber auch andere Faktoren wie saisonale hydrologische Schwankungen, der Einfluss eines nahegelegenen Oberflächengewässers und die landwirtschaftliche Nutzung maßgeblich bei Brielmann et al. (2009).

Säulenversuch

Mit Standortmaterial gefüllte Sedimentsäulen wurden in einer Temperaturorgel inkubiert und kontinuierlich mit sauerstoffreichem ($8,5 \pm 0,5$ $mg \cdot l^{-1}$) Wasser aus einem quartären Karbonatgrundwasserleiter (pH $7,44 \pm 0,8$) durchströmt. Das Wasser wies, wie direkt am Standort der Feldstudie, sehr geringe Konzentrationen an DOC ($0,95 \pm 0,23$ $mg \cdot l^{-1}$), PO_4^{3-} (32 ± 13 $\mu g \cdot l^{-1} P$) und NO_3^- ($7,61 \pm 0,27$ $mg \cdot l^{-1}$) auf. Mittlere Konzentrationen der untersuchten hydrochemischen Parameter beider Wässer sind in Tabelle 1 zusammengefasst. Ein signifikanter Einfluss der Temperatur auf die untersuchten hydrochemischen Parame-

ter wurde auch in dieser Versuchsreihe nicht festgestellt. Der pH-Wert und die Sauerstoffkonzentration konnten allerdings zunächst nur in der Sammelprobe des jeweiligen Säulenauslasses ohne temperaturspezifische Kalibrierung bestimmt werden, was die Aussagefähigkeit der Parameter beeinträchtigte. In einem späteren Versuch konnten beide Parameter im Durchfluss bestimmt werden. Mit steigender Temperatur nahmen sowohl der Sauerstoffgehalt als auch der pH-Wert ab, beides bekannte Phänomene (Balke 1978, Stumm & Morgan 1995) (Daten nicht gezeigt).

Die Bakterienzahl im Abfluss der Säulen variierte zwischen $1{,}8 \cdot 10^4$ und $1{,}1 \cdot 10^5$ Zellen $\cdot\, cm^{-3}$. Um eine direkte Vergleichbarkeit der Daten zu gewährleisten, sind die Ergebnisse aus den Säulenversuchen in Kubikzentimeter je Sedimentsäulenvolumen angegeben, gleichermaßen für das Wasser und das Sediment. Den Berechnungen liegt eine, durch entsprechende volumetrische und gravimetrische Messungen bestimmte, Porosität von 39 % zugrunde, d. h. ein cm^3 Sediment enthält 390 µl Porenwasser. Im Vergleich zur Referenztemperatur von 10 °C waren die Zellzahlen im Säulenwasser bei 20 °C signifikant erhöht (einseitige ANOVA, $p \leq 0{,}001$) (Abb. 5A). Die im Wasser gemessenen Zellzahlen korrelierten zudem signifikant mit der im Säulenwasser bestimmten mikrobiellen Diversität (Spearman's $\rho = 0{,}94$, $p = 0{,}017$), sodass mit zunehmender Zellzahl auch die Diversität zunahm. Im Gegensatz dazu gab es bei den im Sediment bestimmten Zellzahlen diese Zusammenhänge nicht. Die Bakterienzahl im Sediment variierte zwischen $5{,}4 \cdot 10^6$ und $1{,}1 \cdot 10^7$ Zellen $\cdot\, cm^{-3}$ und lag somit um etwa zwei Größenordnungen höher als im Sedimentporenwasser.

Die Phosphataseaktivität (EPA) zeigte sowohl für das Säulenwasser als auch für das Sediment eine starke Abhängigkeit von der Temperatur (Abb. 5B). Im Säulenwasser variierte sie zwischen 1,8 und 23,2 pmol $\cdot\, cm^{-3} \cdot h^{-1}$ und war im Vergleich zur Referenztemperatur (10 °C) bei 45 °C signifikant erhöht. Im Sediment gemessene Phosphataseaktivität schwankte zwischen 0,9 und 8,2 nmol $\cdot\, cm^3 \cdot h^{-1}$, mit signifikant erhöhten Werten bei 20, 30 und 45 °C (einseitige ANOVA, $p \leq 0{,}001$). Die Phosphataseaktivitäten waren im Sediment durchschnittlich um drei Größenordnungen höher als im Säulenwasser.

Die über die bakterielle Aufnahme von ^{3}H-markiertem Thymidin in die DNA ermittelte Kohlenstoffproduktion variierte im Säulenwasser zwischen 0,04 und 0,12 pg C $cm^{-3} \cdot h^{-1}$ (Abb. 5C). Im Sediment konnte eine deutliche Temperaturabhängigkeit der bakteriellen Kohlenstoffproduktion (BKP) nachgewiesen werden. Generell lag die BKP im Sediment zwischen 3,6 und 10,7 pg $\cdot$ C $\cdot\, cm^{-3} \cdot h^{-1}$, mit signifikant niedrigeren Werten bei 4 °C und 45 °C (jeweils −52 %), sowie signifikant erhöhten Werten bei 20 (+41 %) und 30 °C (+37 %) (Abb. 5C).

Die bakterielle Diversität nach Shannon-Wiener H' variierte zwischen 3,0 und 3,4 im Säulenwasser ohne signifikante Korrelation zur Temperatur. Im Säulensediment lag

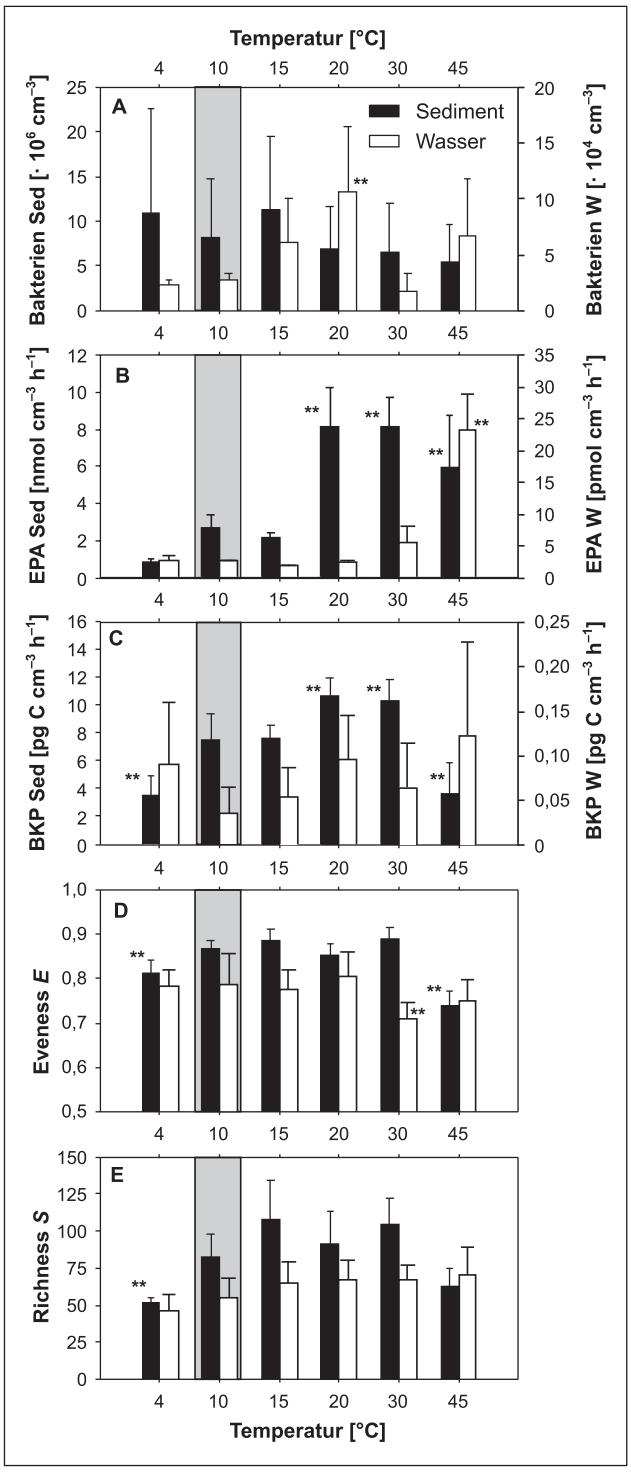

Abb. 5 Temperaturabhängigkeit ausgewählter mikrobiologischer Parameter (Bakterienzahl, extrazelluläre Phosphataseaktivität (EPA), Bakterielle Kohlenstoffproduktion (BKP), Evenness und Richness) im Säulenwasser (weiße Balken) und Säulensediment (schwarze Balken). Daten sind als Mittelwerte (± Standardabweichung) aus 6 Säulenreplikaten gegeben. ** kennzeichnet die von der Referenztemperatur signifikant verschiedenen Werte ($p < 0{,}001$)

die mikrobielle Diversität zwischen 3,1 und 4,1, mit signifikant niedrigeren Werten bei 4 (−16 %) und 45 °C (−18 %) (Daten nicht gezeigt). Im untersuchten System lagen die Werte für die Evenness E im Grundwasser zwischen 0,71 und 0,79, im Sediment zwischen 0,73 und 0,89. Auch für diesen Parameter wurde eine signifikante Verringerung bei 4 °C und 45 °C gegenüber der Referenztemperatur gefunden (Abb. 5D), die folglich auch in der Gesamtzahl der Taxa (Richness S, Taxonomische Einheiten; hier T-RFs) reflektiert wird (Abb. 5E).

Der Einfluss der Temperatur auf die Zusammensetzung der bakteriellen Gemeinschaften konnte sowohl im Säulenwasser als auch im Sediment (MANOVA, $R = 0{,}82$ und $R = 0{,}92$, $p < 0{,}005$) nachgewiesen werden. Die Auswertung der multivariaten Regressionsbäume zeigte vor allem für das Sediment eine deutliche temperaturabhängige Strukturierung der bakteriellen Gemeinschaften (Abb. 6A). So wurden die Gemeinschaften im Sediment bei 4 °C und 45 °C als deutlich verschieden von den Gemeinschaften bei 10, 15, 20 bzw. 30 °C identifiziert, während geringere Unterschiede in der Zusammensetzung der Bakterien im Bereich von 10 bis 30 °C festgestellt wurden. Typische T-RFs für die jeweilige Versuchstemperatur, so z. B. die T-RFs 129, 469, 126, 467 bei 45 °C und die T-RFs 401, 437 und 147 bei 4 °C konnten identifiziert werden (Abb. 6A). Eine Feststellung der durch diese T-RFs repräsentierten Bakterientaxa war im Rahmen dieser Untersuchungen nicht möglich. Auch im Säulenwasser wurden einige temperaturspezifische Indikator-T-RFs wiedergefunden, allerdings war die erklärte Gesamtvarianz und damit die Aussagekraft der multivariaten Regressionsbäume für das Säulenwasser etwas geringer (Abb. 6B).

Temperaturtoleranz von Grundwasserinvertebraten

Individuen von *Niphargus inopinatus* (Grundwasserflohkrebs) zeigten in der Temperaturgradientenkammer deutliche Verteilungsmuster. In mehr als 30 % der Beobachtungen befanden sich die Tiere im Bereich von 12 bis 14 °C und in 77 % der Fälle zwischen 8 und 16 °C (Abb. 7); die mittlere Aufenthaltstemperatur betrug 11,7 ± 3,4 °C. Wiederholt fanden sich Tiere auch in einer Art Kältestarre bei Temperaturen ≤5 °C. Alle Individuen konnten aber, setzte man sie zurück in 12 °C temperiertes Wasser, ohne offensichtliche Folgeschäden, wiederbelebt werden. Kontrollversuche in einer Temperaturkammer ohne Temperaturgradient (einheitlich 12–13 °C) zeigten eine mehr oder weniger gleichmäßige Verteilung der Tiere über die ganze Kammer mit einer signifikanten Anhäufung am unteren Ende der Kammer (34 % der beobachteten Tiere). Der Grund dafür dürfte vor allem eine positive Gravitaxis der Tiere sein und der Umstand, dass dieser Ort den besten Schutz vor Licht bot, welches während der Zählungen eingesetzt wurde. Ein ähnliches Ergebnis lieferten wiederholte Versuche mit der Grundwasserassel *Proasellus cavaticus*. 66 % aller Beobachtungen zeigten die Tiere bei Temperaturen zwischen 8 und 16 °C, insgesamt 24 % bei einer Temperatur von 12–14 °C (Abb. 7); die mittlere Aufenthaltstemperatur betrug 11,4 ± 5 °C. Auch in diesen Versuchen wurde ein Individuum in Kältestarre vorgefunden (bei 2,3 °C), konnte aber erfolgreich reaktiviert werden. Drei weitere Tiere verfielen jedoch bei Temperaturen von 22,9, 23,5 und 25 °C in eine Wärmestarre. Zwei der Tiere konnten bei kühleren Temperaturen wieder aktiviert werden, starben jedoch wenige Stunden bzw. Tage später. Vergleichbar zu *N. inopinatus* verteilten sich auch die Asseln in den Kontrollexperimenten sehr gleichmäßig über die Kammer, mit einer etwas erhöhten Aufenthaltswahrscheinlichkeit an den beiden Kammerenden.

Um die Temperaturtoleranz ausgewählter Grundwasserinvertebraten genauer zu untersuchen, wurden Temperaturversuche im Stil klassischer Tox-Tests durchgeführt. In diesen Versuchen zeigte sich der Grundwasserflohkrebs *N. inopinatus* temperaturtoleranter als die Assel *P. cavaticus*. Nach 24 h betrug die Temperatur, bei der 50 % der Individuen starben (LT_{50}) 27,1 ± 0,5 °C. Nach 48 h waren alle bei 27 bzw. 30 °C inkubierten Individuen gestorben und die LT_{50} sank auf 23,3 ± 2,9 °C. Am Tag 25 und 30 senkte sich die LT_{50} auf 20,2 ± 1,2 °C (Abb. 8A). Nach 24 h wurde für *P. cavaticus* eine LT_{50} von 23 ± 0,1 °C bestimmt, die eine steil abnehmende Tendenz mit der Zeit zeigte. Nach 48 h war der Wert bereits auf 18,8 ± 0,4 °C, nach 96 h auf 17,9 ± 0,6 °C, und nach 5 Tagen schließlich auf 16,6 ± 3,8 °C gesunken (Abb. 8B).

Diskussion

Grundwasserqualität

In zahlreichen Studien konnte gezeigt werden, dass die Abgabe von Wärme in den Untergrund eine Reihe geochemischer Reaktionen maßgeblich beeinflusst (Griffioen & Appelo 1993, Brons et al. 1991, Stumm & Morgan 1995, Arning et al. 2006). Im Gegensatz dazu zeigen die Ergebnisse der vorgestellten Feldstudie und der Säulenexperimente signifikante Veränderungen der Wasserchemie mit der Temperatur nur in Bezug auf die Sauerstoffkonzentration und den pH-Wert. Anscheinend waren die im Feld beobachteten Temperaturerhöhungen zu gering, um chemische Prozesse signifikant zu beeinflussen. Außerdem war die Reaktivität der biologischen Prozesse im Feld und in den Säulenversuchen vermutlich durch die starke Energielimitierung beider Systeme begrenzt. Für „sauberes“ Grundwasser lässt sich somit aus unseren Ergebnissen keine unmittelbare Gefährdung der Grundwasserqualität durch Temperaturveränderungen innerhalb der im Feld untersuchten Spannbreite ableiten.

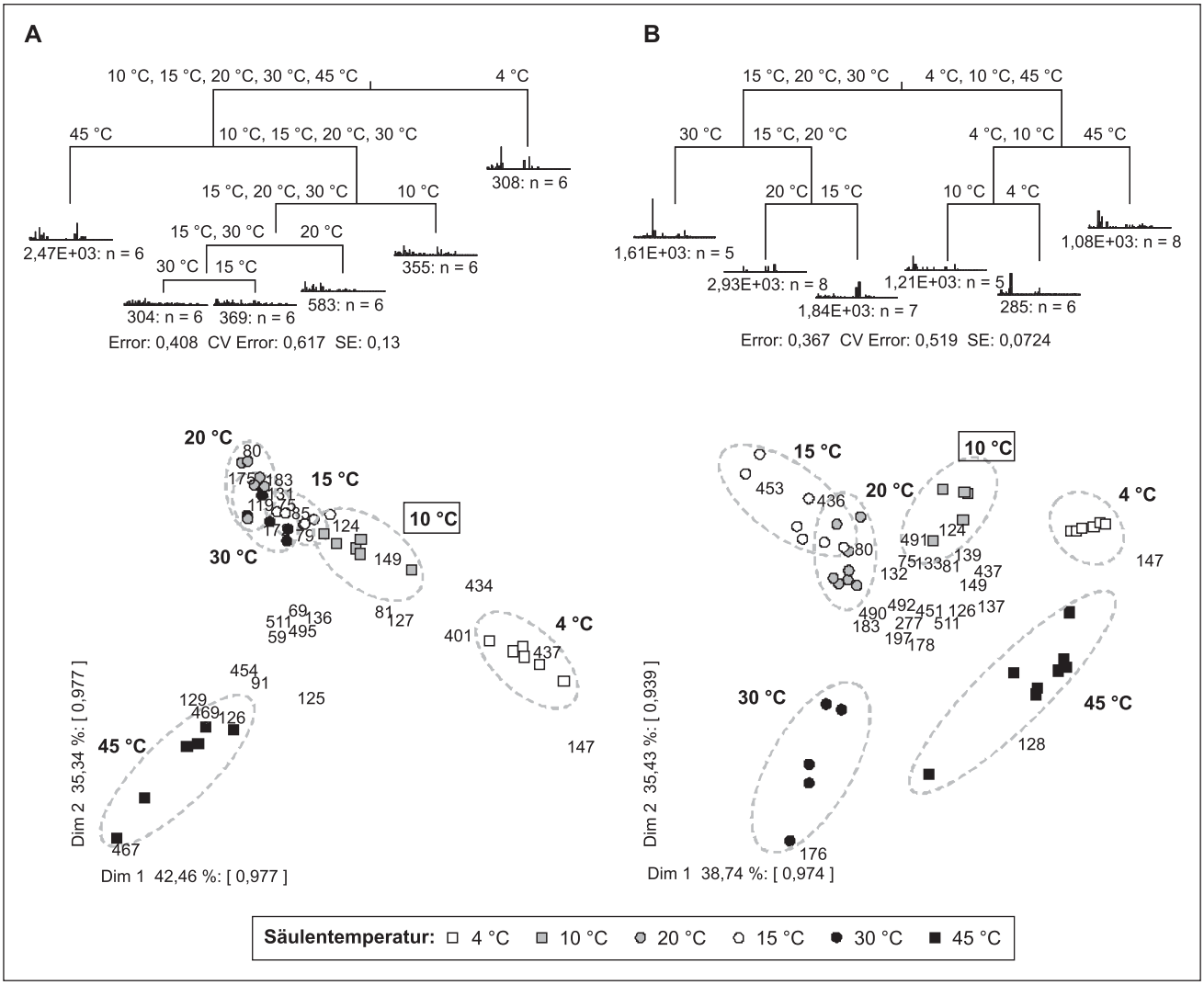

Abb. 6 Multivariate Regressionsbäume (oben) der relativen Abundanzen von Bakterien-T-RFs im Säulensediment (**A**) und Säulenwasser (**B**). Die Unterteilung basiert auf Euklidischen Abständen. Balkendiagramme repräsentieren die mittlere T-RF-Zusammensetzung an jedem Knoten. Ebenfalls dargestellt (*unten*) ist die Hauptkomponentenanalyse der aus den multivariaten Regressionsbäumen resultierenden Gruppen. Die einzelnen Säulen mit ihrer Versuchstemperatur sind durch Symbole (siehe Legende) gekennzeichnet, die Zahlen geben spezifische T-RF's wieder. Allen Gruppen gemeinsame T-RF's wurden aus Gründen der Lesbarkeit nicht dargestellt. Die ersten zwei Hauptkomponenten (Dim 1, Dim 2) erfassen 42,5 % und 35,3 % (A) bzw. 38,7 % und 25,4 % (B) der Varianz in den T-RF's

Effekte von Temperaturänderungen auf Grundwasserlebensgemeinschaften

Mikrobiologie

Eine Temperaturerhöhung führt nach üblicher Lehrmeinung zur Erhöhung der Stoffwechselaktivität und Teilungsrate bei Bakterien (Koolman & Röhm 1998). Die metabolische Aktivität mikrobieller Arten hat jedoch individuelle und spezifische Temperaturoptima (Abb. 1). Während eine natürliche Grundwassertemperatur (etwa 10–12 °C) optimale Wachstumsbedingungen für psychrophile und psychrotolerante Mikroorganismen darstellt, fördert eine Temperaturerhöhung auf 15 bis 20 °C bereits mesophile und noch höhere Temperaturen ab 40 °C gar thermophile Arten (Abb. 1).

Eine Veränderung der Wassertemperatur spiegelt sich in der dargestellten Studie nicht gleichermaßen in allen Parametern wider. Während im Grund- und Säulenwasser mikrobielle Abundanzen und Aktivitäten durch die aufgetretenen Temperaturveränderungen und aufgrund der geringen Substrat- und Nährstoffverfügbarkeit entweder gar nicht (Feld) oder nur im vernachlässigbaren Umfang (Säulen) beeinflusst wurden, erwies sich die bakterielle Diversität als temperatursensitiver Parameter. Der Anstieg der Bakteriendiversität innerhalb der untersuchten Temperaturspanne im Feld steht im Einklang mit der „Intermediate Disturbance

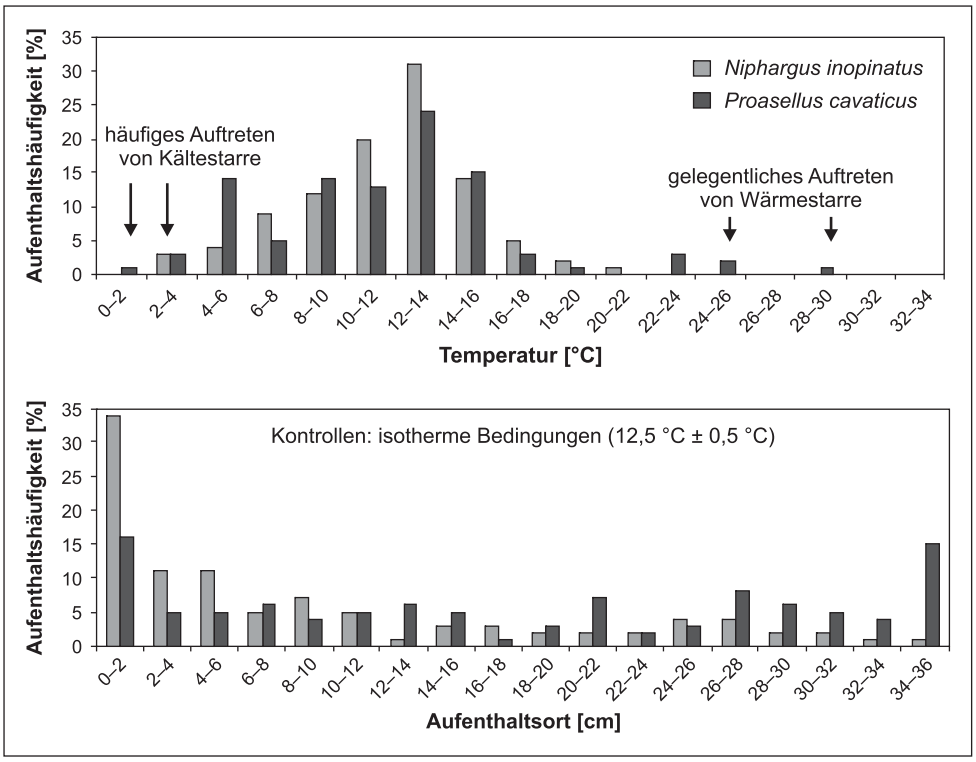

Abb. 7 Aufenthaltshäufigkeiten zweier ausgewählter Grundwasserinvertebraten, *Niphargus inopinatus* ($n = 274$) und *Proasellus cavaticus* ($n = 156$) innerhalb eines Temperaturgradienten über den Beobachtungszeitraum von 24 h (*oben*). Die untere Darstellung zeigt die Verteilungshäufigkeit der Tiere bei isothermen (12,5 °C ±0,5 °C) Bedingungen. Der Zusammenhang zwischen Aufenthaltsort und Temperatur ist aus Abbildung 3 ersichtlich

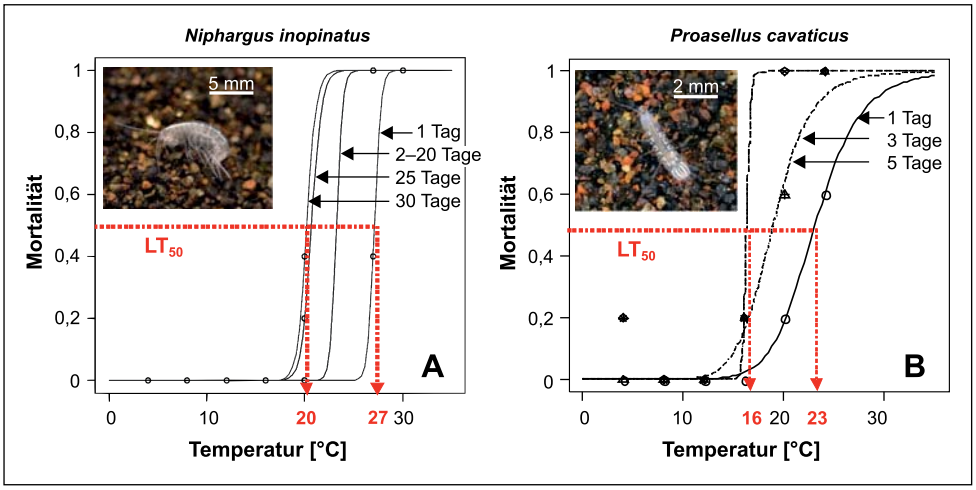

Abb. 8 Temperatur-Dosis-Wirkungs-Beziehungen für zwei ausgewählte Grundwasserinvertebraten. LT_{50} = Letale Temperatur für 50 % der Versuchstiere. Die Versuche wurden dynamisch über einen Zeitraum von 5 Tagen (*P. cavaticus*) bis 30 Tage (*N. inopinatus*) ausgewertet. (**A**) verändert nach Schreglmann (2010), (**B**) verändert nach Ferraro (2009)

Hypothesis“, derzufolge die Artenvielfalt sich bei mäßiger Intensität und Frequenz einer Störung erhöht (Connell 1978, Ward & Stanford 1983, Dial & Roughgarden 1998, Lake 2000).

Zudem werden am Sediment festsitzende und im Grundwasserleiter suspendierte Bakteriengemeinschaften in unterschiedlichem Maße von der Temperatur beeinflusst. Generell leben je nach Nährstoff- und Belastungssituation zwischen 80 und 99,99 % der Zellen im Grundwasserleiter festsitzend (Alfreider et al. 1997, Griebler et al. 2002). In den Säulenversuchen waren zwischen 98,46 % und 99,79 % der Zellen sediment-assoziiert; ein Indiz für die vorherrschenden nährstofflimitierten Bedingungen, unter denen Bakterien bevorzugt am Sediment verweilen (Harvey et al. 1984). Eine Temperaturerhöhung innerhalb des optimalen Bereichs für psychrotolerante und mesophile Mikroorganismen (10 bis 30 °C, Abb. 1) führte im Sediment zur Erhöhung der Kohlenstoffproduktion der Bakterien. Jenseits dieses Temperaturbereichs (bei 4 und 45 °C) war die Bakterienproduktion signifikant erniedrigt. Die Phosphataseaktivität hingegen ist neben der Temperatur vor allem von der Phosphatverfügbarkeit bestimmt (Stibal et al. 2009). So deuten die im Vergleich zur Referenztemperatur signifikant erhöhten Enzymaktivitäten im Sediment der Säulen darauf hin, dass es

bei Temperaturen ≥ 20 °C aufgrund erhöhter Stoffwechselaktivitäten der mikrobiellen Gemeinschaft zu einer Phosphatlimitierung im untersuchten System kam. Auch im Sediment erwiesen sich die bakterielle Diversität, Richness und Evenness als sehr sensitive Parameter für Temperaturveränderungen. Besser als die Diversität gibt dabei die Evenness E (die Gleichmäßigkeit der Abundanzverteilung der Arten) einen Aufschluss über die funktionelle Stabilität und Redundanz innerhalb mikrobieller Gemeinschaften. Im Allgemeinen bedeutet eine niedrige Evenness E, dass die mikrobielle Gemeinschaft von einigen wenigen Arten dominiert wird und somit die Widerstandsfähigkeit solcher Gemeinschaften gegenüber einer Störung (z. B. durch Temperatur oder eine Verunreinigung) von den dominierenden Arten abhängt. Die starke Umstrukturierung der Bakteriengemeinschaften bei 4 und 45 °C und die damit verbundene Abnahme der Diversität, Richness und Evenness verwiesen auf eine vergleichsweise starke Veränderung. Diese war bei 4 °C wahrscheinlich mit der Inaktivierung mesophiler und einer Dominanz psychrophiler oder psychrotoleranter Arten, bei 45 °C mit dem Verlust bzw. der Inaktivierung psychrophiler und der Etablierung thermophiler Arten verbunden. Hinweise auf die temperaturbedingte Veränderung der Zusammensetzung der mikrobiellen Gemeinschaften bis zur Etablierung thermophiler Arten lieferten bereits die Arbeiten von Aragno (1983) und Schippers & Reichling (2006).

Die Versuche haben gezeigt, dass sich Temperaturunterschiede nur selten unmittelbar in den bakteriellen Parametern im Grund- und Säulenwasser widerspiegeln. Dies mag vor allem durch die hohen Abstandsgeschwindigkeiten ($v_a = 18$–$29\ \mathrm{m \cdot d^{-1}}$) und die dadurch geringen Verweilzeiten des Grundwassers im untersuchten Kiesaquifer und im Säulenexperiment (Verweilzeit ∼13 min) verursacht sein. Bakterien im Wasser sind den Temperaturveränderungen – ob Erhöhung oder Abkühlung – somit zeitlich nur sehr begrenzt ausgesetzt, während festsitzende Gemeinschaften einer längerfristigen Beeinflussung unterliegen. Eine Wasserbeprobung allein liefert daher nicht immer belastbare Aussagen über den Zustand des Grundwasserökosystems. Die Entnahme von Sedimenten im Feld ist allerdings arbeits- und kostenintensiv und bleibt von einem standardisierten Monitoring bei geothermischen Anlagen bislang ausgeschlossen.

Weitere Effekte auf die Mikrobiologie, die im Zusammenhang mit einer Temperaturerhöhung im Grundwasser immer wieder diskutiert werden, sind die starke Schleimproduktion und Verstopfung durch verstärktes Bakterienwachstum und die Gefahr der Verkeimung (Wagner et al. 1988). Während die Gefahr einer Massenentwicklung von Bakterien und einer daraus resultierenden Verstopfung des Grundwasserleiters in organisch unbelasteten Grundwassersystemen gering scheint, kann in Aquiferen mit entsprechender Hintergrundbelastung vermehrtes Bakterienwachstum durchaus auftreten (Alexander 1982, Pagni 1985). Eigene Untersuchungen zeigten, dass insbesondere der Sauerstoffgehalt im Grundwasser bei einer moderat erhöhten DOC-Konzentration (Erhöhung um 3 $\mathrm{mg \cdot l^{-1}}$ bei 1,5 $\mathrm{mg \cdot l^{-1}}$ Hintergrund) rasch abnimmt bei einer gleichzeitigen Erhöhung der Bakterienzahlen und -aktivitäten (unpubl. Daten).

Umgekehrt kann eine Vermehrung von Mikroorganismen auch den Betrieb geothermischer Anlagen beeinträchtigen (Lerm et al. 2011). Da in Grundwasserökosystemen pathogene Keime vorkommen können, besteht die Möglichkeit, dass sich diese bei einer Temperaturerhöhung vermehren (Seppänen in Wagner et al. 1988). Generell überleben pathogene Bakterien und Viren in der Umwelt länger bei niedrigen Temperaturen (Bogosian et al. 1996, Rozen & Belkin 2001), und eine Vermehrung wurde bisher nur in Einzelfällen dokumentiert (Camper et al. 1991). Eigene Untersuchungen in Sedimentsäulen zeigten eine längere Nachweisbarkeit von *Escherichia coli* (als koloniebildende Einheiten [KBE] auf Platten mit Selektivmedium) bei Temperaturen ≤10 °C im Vergleich zu erhöhten Temperaturen (unpubl. Daten). Vital et al. (2007, 2008) haben erst kürzlich gezeigt, dass sich *Vibrio cholerae* (Stamm O1 Ogawa Eltor) und *E. coli* (Stamm O157) in Fluss- und Teichwasser vermehren konnten. Die Wachstumsraten zeigten eine positive Korrelation mit der Temperatur bis 30 °C.

Fauna

Ein niedriger Basisstoffwechsel, geringe Reproduktionsraten und hohe Hungertoleranz sind charakteristisch für die Grundwasserfauna in ihrer im Allgemeinen temperaturkonstanten (10–12 °C), nahrungsarmen Umwelt (Thulin & Hahn 2008). Während die oberirdisch lebende Wasserassel (*Asellus aquaticus*) nur etwa 1 Jahr alt wird, leben manche Grundwasserarten um das 5- bis 10fache länger (Griebler & Mösslacher 2003). In Bezug auf ihre Temperaturtoleranz werden höhere Organismen als *stenotherm* (enger Toleranzbereich) und *eurytherm* (breites Temperaturspektrum) unterschieden. Vertreter der europäischen Grundwasserfauna sind zweifellos meist kalt*stenotherm*. So ist z. B. *Niphargus virei* als echter Grundwasserflohkrebs nur in einem sehr engen Temperaturbereich aktiv, während der nah verwandte Bachflohkrebs *Gammarus fossarum* ein sehr breites Spektrum toleriert (Issartel et al. 2005, Abb. 1). Die Auswirkungen einer Temperaturänderung auf Grundwasserorganismen sind bisher kaum dokumentiert. Für den Grundwasserhüpferling *Parastenocaris phyllura* (Copepoda, Harpacticoida) führten moderate Temperaturerhöhungen (um ∼8 °C) bei ausreichendem Nahrungsangebot zwar zur Verkürzung der Gesamtentwicklungszeit, ein Anstieg über eine artspezifisch kritische Temperatur (19 °C) jedoch zum Absterben der Organismen (Glatzel 1990). Unsere aktuellen Ergebnisse zeigen, dass sowohl die bevorzugten Temperaturbereiche als auch die Sensitivität gegenüber einer Grundwassererwärmung bei verschiedenen Organismengruppen und -arten

sehr unterschiedlich ausgeprägt sein können (Abb. 7 und 8). Die Untersuchungen belegen eine gewisse Wärmetoleranz für wenige Tage. Bei einer Temperaturveränderung im Untergrund sollten deshalb in Bezug auf die Grundwasserfauna verschiedene Aspekte beachtet werden. Da Temperaturen über 20 °C für alle bisher getesteten stygobionten Invertebraten in Abhängigkeit von der Versuchsdauer kritisch waren, sollte diese Temperatur beim derzeitigen Wissensstand nicht überschritten und nur zeitlich bzw. räumlich begrenzt realisiert werden.

Vor allem im städtischen Bereich wurden durch die großflächige Versiegelung der Oberfläche und zahlreiche Tiefbauten (z. B. Kellergeschosse, Tiefgaragen, U-Bahntunnel) die natürlichen Fließbedingungen im Aquifer nachhaltig gestört. Die Grundwassertemperatur im städtischen Bereich ist aufgrund kontinuierlicher Wärmeabgabe aus Abwasser- und Fernwärmenetzen meist bereits bis zu 5 °C erhöht (Zhu et al. 2010). Das hat zum einen Konsequenzen für die Nutzung von Grundwasser zur Gebäudeklimatisierung, zum anderen ist die Grundwasserfauna lang anhaltenden Temperaturveränderungen und einer generell herabgesetzten Grundwasserqualität ausgesetzt.

Schlussfolgerungen und Empfehlungen

Aus ökologischer Sicht ist der zunehmende Ausbau erneuerbarer bzw. unerschöpflicher Energie, welche fossile und nukleare Energie ersetzen, zu begrüßen. Andererseits muss dabei der Schutz des Grundwassers als lebenswichtige Ressource für den Menschen und der Schutz von Grundwasserlebensräumen für eine Vielzahl von Organismen bzw. eine ökologische Funktionalität sichergestellt werden. Nach unserem heutigen Kenntnisstand sind dabei insbesondere folgende Punkte von Bedeutung:

- Die maximal genehmigte Temperaturspanne sollte auf den physikalisch, chemischen und biologischen Zustand des jeweiligen Grundwasserleiters abgestimmt sein. Bei moderaten Temperaturveränderungen gibt es derzeit keine gesicherten Hinweise, dass eine lokale thermische Nutzung zu wesentlichen Störungen in unbelasteten Grundwasserökosystemen führt. In Ländern, wie z. B. in der Schweiz oder in Frankreich wurden Temperaturspannen von ±3 K bzw. ±11 K definiert (Hähnlein et al. 2010b). Die in Deutschland übliche Temperaturspanne von ±6 K scheint in Bezug auf unsere bisherigen Untersuchungen vertretbar.
- Wenn Konzentrationen von DOC (>3 mg$\cdot$l^{-1} in oxischen Grundwässern) und Nährstoffen (z. B. $PO_4^{3-} > 0{,}2$ mg$\cdot$l^{-1}) über dem natürlichen Hintergrund liegen (Kunkel et al. 2004) und in typischerweise oxischen Grundwasserleitern nur geringe Sauerstoffkonzentrationen (<3 mg$\cdot$l^{-1}) vorliegen, sollte dieser Wert allerdings einzelfallbezogen geprüft und keinesfalls überschritten werden.
- In organisch belasteten Grundwassersystemen kann eine Temperaturerhöhung rasch zu einer Sauerstoffzehrung führen, die massive Veränderungen innerhalb der mikrobiellen Gemeinschaft zur Folge hat und höheren Organismen kein dauerhaftes Überleben ermöglicht. Auch die Vermehrung von pathogenen Mikroorganismen ist bei erhöhter Temperatur (z. B. Legionellen) und erhöhten DOC- und Nährstoffkonzentrationen (z. B. *Vibrio cholerae*) nicht auszuschließen.
- Ausnahmefälle stellen reduzierte weil organisch belastete Grundwasserleiter dar. Hier könnte die Erdwärmenutzung zu Kühlzwecken und die daraus resultierende Erhöhung der Temperatur im Untergrund zu einem positiven Effekt führen, der erhöhten Mobilisierung von Schadstoffen und dem verstärkten mikrobiellen Schadstoffabbau (*Enhanced Natural Attenuation*). Dies ist im Einzelfall unter Bedacht möglicher negativer Begleiterscheinungen (z. B. Methan- und Sulfidproduktion) durch Vorversuche abzuklären.

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RESEARCH PAPER

Detection of catecholamines in single specimens of groundwater amphipods

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Abstract Catecholamines play essential roles in several physiological processes in vertebrates as well as in invertebrates. While several studies have shown the presence of these substances in surface water invertebrates, their occurrence in groundwater fauna is unproven. In the present study, the presence of different catecholamines (i.e., noradrenaline, adrenaline, and dopamine) in individual specimens of groundwater amphipods of the genus *Niphargus* (mostly *Niphargus inopinatus*) was investigated via two independent analytical methods: HPLC/EcD and UPLC/TOF-MS. Mean values for catecholamine levels were 533 pg mg^{-1} fresh weight for noradrenaline, 314 pg mg^{-1} for adrenaline, and 16.4 ng mg^{-1} for dopamine. The optimized protocol allowed the detection of CAs in single organisms of less than 1 mg fresh weight. Catecholamine concentration patterns in groundwater invertebrates are briefly discussed here with respect to their evolutionary adaptation to an environmentally stable, energy-poor habitat.

Keywords Catecholamine analysis · Dopamine · Adrenaline · Noradrenaline · Groundwater amphipod · *Niphargus*

Introduction

Catecholamines—derivatives of 1,2-dihydroxybenzene (catechol)—are synthesized biogenetically from aromatic amino acids such as L-tyrosine or L-3,4-dihydroxyphenylalanine, and act as hormones and neurotransmitters in organisms. They are found in vertebrates as well as in invertebrates. In both groups of animals, catecholamines (CAs, i.e., noradrenaline, adrenaline, and dopamine) play an essential role [1–7]. However, the neuroendocrine mechanisms that mediate stress response in invertebrates are far less understood than in vertebrates. In invertebrates, an obviously ancestral type of stress response is present [1]. It involves CAs as major messengers, as has been shown for molluscs [3]. Moreover, several processes in molluscs, including feeding [8], locomotion [9], and immunity [4–6] are affected by CAs. In the hemolymph of the scallop, *Chlamys farreri*, an increase in the levels of noradrenaline (NA) and adrenaline (A) was observed in response to environmental stressors such as high temperature, low salinity, and exposure to air [2]. Available information on the occurrence and functions of dopamine (DA) in invertebrates is even scarcer. As shown for *C. elegans*, DA acts both synaptically and extrasynaptically [10], and is involved in modulatory control of egg-laying, defecation, motor activity, response to food, and habituation to touch [11]. Without doubt, catecholamines play a major role in the coordination of invertebrate physiology, and recent studies indicate that CAs are also strongly involved in the stress response of crustaceans. For

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example, Aparicio-Simón et al. [12] analyzed the concentrations of catecholamines in the Pacific whiteleg shrimp *Litopenaeus vannamei* during handling stress. They observed changes in CA levels in several tissues related to a set of metabolic changes, thus concluding that CAs possibly act as mediators of the primary stress response. Considering this evidence, as well as the studies mentioned above, we assume that catecholamines are potential stress indicator compounds. However, if we consider groundwater invertebrates (stygobionts), not even the mere presence of catecholamines has been investigated so far.

Groundwater ecosystems differ from surface environments in many aspects. They are perceived as energy-limited habitats that are devoid of light and experience harsh but stable environmental conditions [13]. The temperature in groundwater in the temperate regions is fairly low, for example 10–12 °C in Germany, reflecting the yearly mean air temperature value. As a consequence, groundwater invertebrates are assumed to be particularly vulnerable to disturbance and stress, such as those caused by organic and inorganic pollution [14–16] or changes in temperature [17]. Since groundwater in Europe and other parts of the world is the most important source of water, a water quality and ecosystem status assessment methodology based on stress biomarkers in invertebrates constitutes a promising approach. Understanding the stress response in these organisms may thus allow the impacts of various kinds of contaminations to be evaluated. Moreover, CAs could probably also be used as sublethal endpoints in acute ecotoxicological bioassays.

The most important group of groundwater invertebrates are the crustaceans, which generally account for ≥50 % of species and specimens [18–20]. Among these, the amphipods comprise a widely distributed group that represent the top consumers in aquifers. With over 300 species and subspecies described, *Niphargus* is the most prominent genus of freshwater amphipods [21]. Species of *Niphargus* are found throughout central and particularly southeastern Europe, where they exhibit high levels of endemism in karst systems [22, 23]. Consequently, we consider niphargids to be ideal model organisms for studying subterranean invertebrates.

In most of the reports on CAs in invertebrates published so far, CAs have been analyzed qualitatively using histochemical methods [24, 25] or quantitatively via high-performance liquid chromatography coupled with an electrochemical detector (HPLC/EcD) [12, 26, 27]. However, none of the studies mentioned above, while using HPLC, confirmed their results by applying an independent second analytical method. The typically low abundances of groundwater fauna in the field place considerable limitations on the availability of test organisms. Therefore, in groundwater studies it is difficult to conduct measurements with homogenates of several organisms while aiming for a big sample size in the experimental setup. Another challenge is the small size of the organisms of interest, as well as the possible release of CAs from organisms during stress response and sample preparation. So far, stress was evaluated via changes in respiration (oxygen uptake typically measured in respirometers, i.e. small chambers [28]). In some studies, the moving behavior was evaluated in relation to chemical stress [29]. However, to our knowledge, no specific substance indicating stress in groundwater invertebrates has been identified so far. Merely, changes in the activity of the respiratory electron transport systems in epigean and hypogean crustaceans (in response to light) have been tested-by reducing tetrazolium salts (INT) to formazan [30].

In the study reported in this paper, the presence of catecholamines in groundwater invertebrates of the genus *Niphargus* is demonstrated for the first time, and is proved by applying two independent analytical methods: HPLC/EcD (quantitative) and ultra performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC/TOF-MS) (qualitative). The protocol allows the detection of CAs in single individuals of less than 1 mg fresh weight.

Materials and methods

Test organisms

All organisms tested belonged to the genus *Niphargus*. Species in this genus are characterized by extremely diverse morphologies, which makes exact taxonomic classification very difficult [31]. As determination to species level in *Niphargus* requires microscopic examination, assignment of the organisms tested to individual species was not possible prior to catecholamine analysis. Nevertheless, frequent taxonomic analysis of the niphargid species collected from the same groundwater wells during other sampling events confirmed that the dominant species was *Niphargus inopinatus* Schellenberg (Fig. 1), which comprised >95 % of the individuals collected, occasionally accompanied by *Niphargus bajuvaricus* Schellenberg.

Sample collection and preparation

Specimens of *Niphargus* were collected from groundwater monitoring wells at the campus of the Helmholtz Zentrum München during three sampling surveys in January 2010. The wells are situated in a shallow Quaternary porous aquifer, which is part of the Munich gravel plain [32, 33]. The animals were collected from the bottom of the wells using a phreatobiological net sampler (mesh size: 74 μm), transferred into 50 mL Falcon tubes filled with ambient groundwater, and transported to the lab in a cooling box.

Fig. 1 *Niphargus inopinatus* Schellenberg, 4 mm long, photo: Günter Teichmann, Helmholtz Center Munich

Subsequently, each specimen was transferred to a single well of a 6-well plate, which was filled with groundwater. Additionally, each well contained a small amount of sediment and detritus particles obtained together with the animals. The 6-well plates were kept in the dark at a temperature of 10 °C for at least 4 weeks to allow the amphipods to acclimatize to lab conditions. In preparation for catecholamine analysis, each amphipod was transferred into a separate 2 mL conical polypropylene (PP) micro test tube containing 1 mL of groundwater (10 °C). In order to assess the basal catecholamine levels that occur in groundwater organisms under near-undisturbed conditions, it was crucial that the animals did not suffer any disturbances, including agitation due to handling procedures, that could lead to stress or panic reactions. Thus, after transferring the organisms into the tubes, they were allowed to rest for 2 h without any disturbance. Following this, each tube was shock-frozen in liquid nitrogen for 20 s, which was sufficient to completely freeze the water pocket, including the animal. The tubes were then left to thaw at room temperature, and subsequently 250 μL of a 10 g/L sodium pyrosulfite solution (antioxidant) were added. All tubes were briefly vortexed and then stored at −80 °C until the extraction and analysis of catecholamines.

In order to calculate mass-specific catecholamine concentrations, the measured CA values for each individual were divided by 0.7 mg. This was the mean fresh weight of 22 additional individuals of a similar size and from the same sampling location that were not used in the analysis. The specimens analyzed for CAs were not weighed in order to avoid possible losses of CAs due to the handling procedure.

A total of nine samples, consisting of six single samples each with one individual and three pooled samples each with three individuals, were analyzed via HPLC/EcD by separating the adherent water and the animal. As some catecholamines had most probably leached into the water due to repeated defrosting, potentially causing damage to the cells, the values determined for water and animal tissue were summed in order to obtain the total concentration of CAs per individual. Additionally, 11 more samples were analyzed via HPLC/EcD by combining the adherent water and the animal. These later samples contained only one individual. Three of these samples were also analyzed via UPLC/TOF-MS for additional verification of the chemical identity of the chromatogram peak attributed (based on the retention time in HPLC/EcD) to dopamine.

The following preparatory steps were based on materials from and adapted procedures for the ClinRep® Complete Kit for Catecholamines in Plasma and the appendant instructions for determining catecholamines in plasma by HPLC [34]. Prior to analysis, the samples were defrosted and, for nine samples, the water was removed from the microtubes and analyzed separately. The following solutions were then added to each sample: 1 mL of TRIS buffer (2 M, pH 8.5, order no. 1072, RECIPE®); 200 μL of internal standard (IS) (10 pg/μL DHBA, order no. 1012, RECIPE®); 100 μL of a stock solution of sodium pyrosulfite ($Na_2S_2O_5$, 5 g/L) in TRIS buffer. Subsequently, the samples were homogenized for 40 s using an ultrasonic homogenizer with a micro-sonotrode tip (2 mm OD; Bandelin Electronics, Berlin, Germany). During homogenization, all of the samples were kept on ice. The influence of ultrasonic homogenization on catecholamine concentrations was tested in preliminary experiments, and was found to be negligible (see Table S1 in the "Electronic supplementary material," ESM).

The homogenized suspension was centrifuged for 2 min at 1800×*g* (Galaxy Mini microcentrifuge, VWR, Radnor, PA, USA), and the supernatant was subsequently transferred into sample preparation columns that were capped at the bottom and contained an alumina suspension in TRIS buffer (order no. 1020, RECIPE®). To bind the substances selectively onto the alumina, the cartridges were vortexed for 10 min (Vortex, Heidolph, Schwabach, Germany). Then a vacuum manifold (Visiprep DL, Supelco, Bellefonte, PA, USA) was used to evacuate the sample preparation columns after removing the bottom cap. The remaining alumina layer was washed three times with 1 mL of added washing solution (order no. 1021, RECIPE®), again shaking the bottom-capped cartridges by hand for 10 s each time, before being sucked empty with the vacuum manifold.

After evacuation, the cap on the bottom of the sample preparation column was replaced with a 250-μL PP elution vial (order no. 1061, RECIPE®) and 120 μL eluting reagent (order no. 1022, RECIPE®) were added. Subsequently, the columns were vortexed for 30 s and the eluting reagent was centrifuged at 600×*g* into the elution tube (centrifuge, Hettich Universal, Newport Pagnell, UK). After transferring the eluent into HPLC vials (PP, conical, 300 μL), 40 μL of the sample were injected into the HPLC system.

After measuring samples 1–11, 17, 18, 21–22, the water in which the *Niphargus* individuals had been frozen was prepared and measured. In each case, 1 mL of this water was transferred into the sample preparation columns and 200 μL IS were added. As a homogenization step was unnecessary, the columns were then directly vortexed to bind the catecholamines. The following preparatory steps were identical to the procedure described above.

After finding a considerable amount of catecholamines in the isolated water, we decided to measure the remaining samples without separating the water from the solid. Therefore, the water and animals in samples 12–14, 16, 19, 20, and 24–27 were prepared together. To do this, 200 μL IS were added to the defrosted sample, which was then homogenized and processed according to the description above.

CAs are susceptible to oxidation. Therefore, these substances, as well as any unprocessed samples, should be stored at low temperatures (ideally +4 °C) and protected from light. During the course of sample processing, the antioxidant sodium pyrosulfite was used to inhibit or decelerate the oxidation process. The storage lifetime of eluted samples is 24 h at room temperature. To facilitate longer storage times, the samples should be kept at −20 °C or less. However, in order to avoid losses, the samples should not be thawed and refrozen repeatedly.

Preparation of a *Niphargus* homogenate for the pooling experiments and preparation of standard stock solution

Frozen *Niphargus inopinatus* were separated from the surrounding water and transferred into a beaker containing 0.3 mL TRIS buffer and 0.1 mL aqueous sodium disulfite solution (5 g/L) per *Niphargus* individual. The mixture was homogenized on ice (10 times, 40 s, 1 min cooling break) using an ultrasonic homogenizer, as above. Then, 0.7 mL TRIS buffer per individual were added, the homogenate was divided into equal aliquots (V=1.1 mL), and these were frozen at –80 °C. Each aliquot represents, on average, the matrix of one *Niphargus*.

Standard stock solutions (noradrenaline hydrochloride, Fluka, Buchs, Switzerland; dopamine hydrochloride, Sigma, St. Louis, MO, USA; adrenaline, Sigma) were prepared in 1 M hydrochloric acid and stored at 4 °C [35].

Analysis of catecholamines

HPLC/EcD analysis

The catecholamines were analyzed by a Dionex HPLC system (with a GP40 pump; Dionex, Sunnyvale, CA, USA) coupled to an electrochemical detection system with a glassy carbon electrode (ED40, Dionex). Forty microliters of extracted catecholamines were injected using an AS50 autosampler (Dionex) with an automatic injection valve (100-μL loop). Between injections, the syringe and loop were flushed with 10 % methanol in distilled water in order to prevent contaminations. Analytes were separated on a C-18-based reversed-phase analytical column (4.6×150 mm) for catecholamines in blood plasma (order no. 1030, RECIPE®). For the analysis, isocratic elution was used (mobile phase, order no. 1210, RECIPE®) at a constant flow rate of 1 mL/min. The column compartment was set to 30 °C, and the detector potential to 700 mV against Ag/AgCl. The catecholamines were identified by comparing the retention times to those of known standards, and quantified using the peak area ratio in relation to the internal standard DHBA (software: Peaknet 5.2, 1992–2000 Dionex).

Calibration

The system was calibrated by threefold injection of 10 μL of standard solution (order no. 1011, RECIPE®) with known concentrations of catecholamines (NA 10 pg/μL, A 6 pg/μL, IS DHBA 10 pg/μL, DA 6 pg/μL), according to the instructions of the kit. The linearity of the CA calibration over a wide concentration range was examined by performing three injections per sample (40 μL per injection to cover the wide range of injected total amounts, to avoid excessive maximum concentrations, and to align with the default injection volume of the samples) of self-prepared standard solutions (combinations of A, NA, and DA; 50, 100, 200, 800, 2000 and 10,000 pg/sample; 1 M HCl, like the commercial standard). The coefficients of determination (R^2) of the calibration curves indicated that they were linear (DA R^2=0.9532; NA R^2=0.9795, and A R^2=0.9726) up to at least a CA concentration of 10,000 pg/sample (Table S2, Fig. S1 in the ESM). The somewhat worse R^2 value for DA may be explained by the relatively broad nature of the peaks it presents in chromatography, which increases integration error, especially at low concentrations.

The effect of using different injection volumes (V= 10 μL, V=40 μL) for the default calibration and the sample injection was examined. The total differences between the CA/IS area ratios ranged from 1.52 % for NA/IS to 5.26 % for A/IS to the maximum of 6.38 % for DA/IS, indicating a negligible influence on the measurement results.

Quality assurance

The long-term quality of the HPLC/EcD calibration was checked by comparing 13 calibrations (injecting 10 μL of the standard solution, RECIPE®) in a control card. The mean peak area and standard deviation for each CA

response was calculated based on measurements performed over a period of 3 weeks. Figures S2 and S3 (in the ESM) show that all of the CA ratios in this time period are within the range of ± two standard deviations, so the data from the HPLC/EcD measurements are comparable over a long time period.

An LOD (limit of detection) test with different dilutions of the standard mixture (RECIPE®) was also conducted. The detection limit was 0.33 pg/μL for NA, 0.2 pg/μL A, and 0.33 pg/μL DA (injection volume 10 μL).

The precision and accuracy of the mean for the method was evaluated by injecting the same CA concentration (diluted catecholamine standard, part no. 45-0206, Thermo Scientific, Waltham, MA, USA; nominal 800 pg/sample, injection volume=40 μL, 1 M HCl) 12 times. The imprecision and measurement deviation (bias) were found to be 11.00 % (bias 9.59 %) for NA, 11.63 % (bias 13.44 %) for A, and 10.00 % (bias 2.62 %) for DA. According to MacDonald [36], both quality parameters can be merged into one characteristic parameter, the (relative) root mean square of the deviation of measurement (RMSD). The corresponding values for NA, A, and DA were 13.52 %, 16.54 %, and 10.16 % respectively.

To evaluate the quality of the data obtained when real sample matrices were analyzed, the precision of the method used and the measurements obtained with HPLC/EcD was determined by performing triplicate measurements of 7 aliquot samples from 14 pooled and homogenized *Niphargus* with measured native mean CA concentrations of 16.5, 31.5, and 174.4 pg/sample for NA, A, and DA. Standard addition (222.5, 388.8, and 376.8 pg/sample NA, A, and DA, respectively) was applied to ensure that measurements were performed in the linear range of the HPLC/EcD. The concentration of DHBA was 2000 pg/sample in all samples. The resulting imprecisions were 15.2 % for NA, 17.3 % for A, and 9.9 % for DA (Fig. S4 in the ESM).

A calibration curve based on a homogenate of *Niphargus* (N=10, CA concentration=10–50 000 pg/sample) showed that the CAs could be measured in their linear ranges [DA (R^2=0.9965), NA (R^2=0.9991) and A (R^2=0.9987)] up to the highest sample concentrations (see Fig. S5 in the ESM).

Derivatization of selected *Niphargus* extracts with AccQ•Tag

To 40 μL of the freshly prepared sample extract in a vial (with the exception of sample 27, where 20 μL extract were used), 70 μL of borate buffer (AccQ•Tag reagent 1, AccQ reagent kit, Waters, Milford, MA, USA) were added and vortexed for 10 s, and then 20 μL of 9 μg/μL 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate in acetonitrile (AccQ•Tag reagent 2, AccQ reagent kit, Waters) were added, vortexed for 10 s, and kept at room temperature for 1 min. The closed vial was then heated to 55 °C for 10 min, and the sample was ready for analysis after cooling.

UPLC/MS analysis

Using a NanoAcquity UPLC system (Waters Micromass, Manchester, UK), an aliquot of 1 μL of sample was injected via a trap column (Symmetry C-18, 180 μm×20 mm, particle size 5 μm, Waters) at 4 μL/min acetonitrile/water 5:95 v/v, 0.1 % formic acid (eluent A), trapping time 4 min, onto a HydroSphere C18 nano-HPLC column, 75 μm×150 mm, particle size 3 μm (YMC Europe, Dinslaken, Germany). Separation was performed at 0.3 μL/min and 40 °C with an initial mobile phase of 100 % eluent A for 3 min. The subsequent gradient elution changed the composition to 100 % B (acetonitrile, 0.1 % formic acid) within 5 min, with this composition held for 12 min. Then the initial conditions were re-established, followed by an equilibration time of 6 min until the end of the cycle after 26 min in total.

The UPLC eluent was introduced into the nanospray source of a Q-TOF 2 mass spectrometer (Micromass) for positive electrospray ionization (ESI). The voltage of the PicoTip electrospray emitter (10 μm orifice, New Objective, Woburn, MA, USA) was set to 1.8–2 kV. Further settings were: MS cone voltage 18 V, collision energy 5 eV, collision gas was argon, MCP detector 1.9 kV, and scan time 2 s from m/z 130–500. Analytes were monitored in the extracted mass chromatograms as mono-protonated $[M+H]^+$ molecular ions.

Results and discussion

Catecholamines in *Niphargus*

Our results show that catecholamines exist in *Niphargus* species. The mean levels of catecholamines were 533 pg/mg fresh weight for NA, 314 pg/mg for A, and 16,400 pg/mg for DA. The variations between each two replicate measurements of the same sample were minimal (see Table 1). However, there were large differences between samples, whether these comprised single or pooled individuals. The coefficients of variance were 53 % for NA, 314 % for A, and 57 % for DA. Such high variance is not surprising if we recall that the analysis is mostly based on single individuals rather than homogenates of several specimens. The standard deviation in the adrenaline concentration was especially high for sample 1, which was clearly an outlier (see Table 1).

To investigate whether the CAs in the samples were exclusively from the animals, the ground water that constituted their habitat was measured. Because no CAs were detected in that water, it can be assumed that all of the CAs in the samples originated from the body tissue and fluid of the animals.

Table 1 Catecholamine concentrations measured in *Niphargus* species via HPLC/EcD

Sample	Catecholamine concentration (pg/mg fresh weight)											
	Animal tissue				Water				Total			
	NA	A	DA	R (%)	NA	A	DA	R (%)	NA	A	DA	R (%)
1	nd	4399.0	32.6	49	nd	44.4	36.9	69	nd	4443.4	69.3	–
	88.6	4235.7	44.4	52	nd	46.3	44.1	69	88.6	4282.0	88.6	–
2	220.9	65.1	4618.6	35	330.4	33.9	13560.4	71	551.3	99.0	18179.0	–
	217.3	74.6	4638.3	35	318.4	34.1	13488.9	71	535.7	108.7	18127.1	–
3,4,5[a]	488.9	14.1	8838.7	26	321.9	67.9	7873.1	68	810.7	82.0	16711.9	–
	495.1	nd	8820.1	26	298.1	61.7	7854.0	68	793.4	61.7	16674.0	–
6,7,8[a]	299.7	nd	7250.9	40	284.6	49.6	10516.0	69	584.3	49.6	17766.9	–
	293.7	nd	7370.7	40	290.6	56.0	10468.1	69	584.3	56.0	17838.9	–
9	164.0	nd	4808.0	29	147.1	nd	6261.1	72	311.1	nd	11069.0	–
	140.4	nd	4698.1	29	151.7	nd	6221.3	73	292.3	nd	10919.4	–
10	310.9	79.3	1600.6	29	466.4	208.0	9458.4	71	777.3	287.4	11059.0	–
	298.9	85.7	1605.4	29	469.0	209.1	9376.7	72	767.9	294.9	10982.0	–
11,17,18[a,b]	276.3	86.3	7333.6	23	274.6	51.3	8998.4	64	550.7	137.4	16332.1	–
	276.3	86.3	7333.6	23	269.7	67.1	8991.7	64	545.9	153.3	16325.4	–
12	–	–	–	–	–	–	–	–	639.4	31.3	26684.6	71
	–	–	–	–	–	–	–	–	633.0	27.6	27055.7	70
13	–	–	–	–	–	–	–	–	583.3	17.7	31835.4	73
	–	–	–	–	–	–	–	–	573.4	29.1	31729.1	73
14	–	–	–	–	–	–	–	–	699.7	nd	23004.7	70
	–	–	–	–	–	–	–	–	704.7	44.6	23323.1	69
16	–	–	–	–	–	–	–	–	1199.9	311.9	17921.9	30
	–	–	–	–	–	–	–	–	1181.9	302.1	17896.1	30
19	–	–	–	–	–	–	–	–	373.1	81.6	17692.7	63
	–	–	–	–	–	–	–	–	363.1	77.6	17492.0	63
20	–	–	–	–	–	–	–	–	546.1	70.1	29522.0	61
	–	–	–	–	–	–	–	–	550.6	nd	29657.9	59
21[b]	31.7	nd	103.9	42	nd	37.3	60.1	72	31.7	37.3	163.9	–
	46.7	nd	101.3	43	nd	35.7	53.1	71	46.7	35.7	154.4	–
22[b]	409.0	nd	8038.7	42	129.3	49.1	3236.9	68	538.3	49.1	11275.6	–
	404.6	nd	8066.9	42	133.1	44.3	3216.0	68	537.7	44.3	11282.7	–
23[b]	–	–	–	–	–	–	–	–	36.1	65.7	73.6	61
	–	–	–	–	–	–	–	–	43.9	52.1	83.9	63
24[c]	–	–	–	–	–	–	–	–	647.4	nd	21959.0	21
25[c]	–	–	–	–	–	–	–	–	542.3	63.6	29080.3	57
26[c]	–	–	–	–	–	–	–	–	626.1	230.1	19023.0	41
27[c]	–	–	–	–	–	–	–	–	726.9	nd	18621.6	28
	–	–	–	–	–	–	–	–	692.7	nd	18999.9	24

[a] Animals in these samples were pooled. [b] Animals were observed to swim up to the water surface just before shock-freezing with liquid nitrogen, and therefore could have been slightly disturbed due to handling. [c] Samples were measured only once because of a sample shortage due to the use of aliquots for derivatization and UPLC/TOF-MS experiments. For all calculations, an average fresh weight of 0.7 mg per animal was used (the numbers assigned to the samples do not necessarily reflect the sequence in which they were measured)

Therefore, based on the results we obtained, it is difficult to draw a general conclusion about the basal levels of the catecholamines, especially dopamine, present in *Niphargus inopinatus*. The high variance in the dataset can be at least partly explained by the fact that the animals differed in age, as well as in size and body mass. Moreover, a few

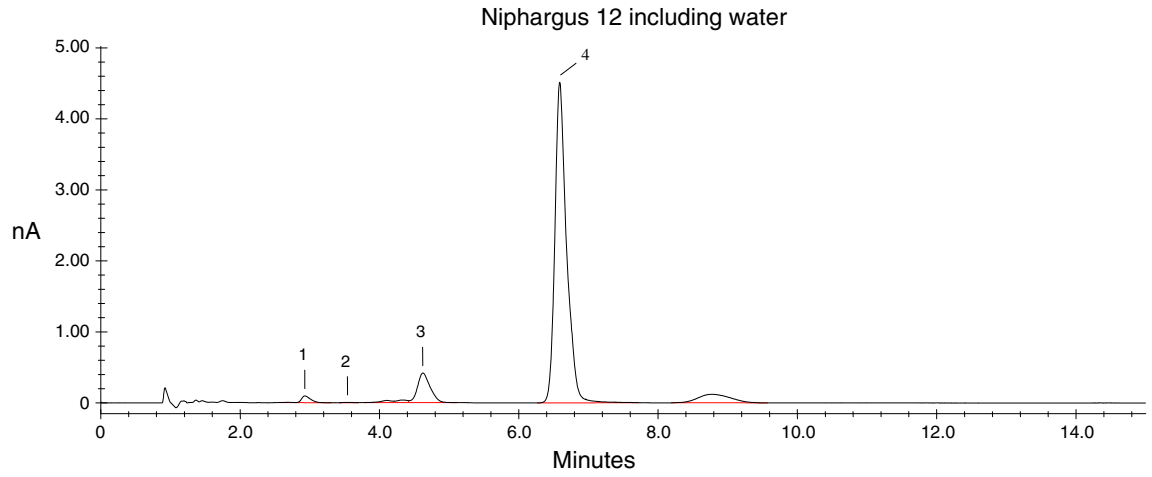

Fig. 2 Chromatogram of catecholamines in a single individual of *Niphargus*, analyzed together with the groundwater in the vial (sample 12): *1* noradrenaline, *2* adrenaline, *3* DHBA, *4* dopamine

individuals of *Niphargus bajuvaricus*—which also occurred in the same habitat, albeit in much smaller numbers—may have been analyzed along with *Niphargus inopinatus*.

It is apparent that the catecholamine levels were highest in the samples that were analyzed together with the groundwater the specimens were frozen in (Fig. 2).

Originally, we expected that we would need to pool several individuals into one sample in order to obtain sufficient amounts of catecholamines above the detection limit. However, it turned out that the dopamine concentrations, even in single individuals, were very high. Therefore, we do not recommend the extraction and analysis of more than one individual at a time, as the results obtained in such a manner could be biased due by measurement inaccuracy at high dopamine levels. Moreover, the occurrence of additional interfering peaks in Fig. 3 with retention times similar to that of DHBA was much more significant in samples where three individuals were analyzed together. Therefore, in this case, the peak area for the internal standard was less accurate.

Along with the collection of animals and the necessary preparation steps, the samples were initially frozen in liquid nitrogen before being thawed and refrozen at −80 °C two more times. We assume that this led to some cell damage and the release of catecholamines into the surrounding water. This point awaits further confirmation. For the moment, we suggest that the animals should be analyzed together with the water they are preserved in (Fig. 4).

There was one obvious outlier in the dataset (sample 1). Compared to the other measurements, the amount of dopamine was rather low and the concentration of adrenaline was especially high. The amount of noradrenaline was below our

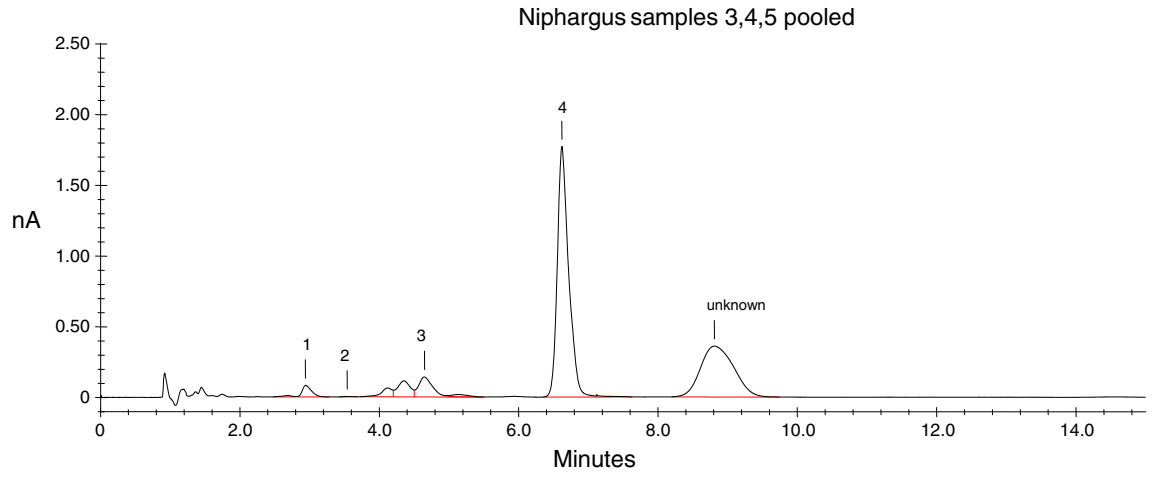

Fig. 3 Chromatogram of catecholamines in a mixed sample of three individuals of *Niphargus* (samples 3, 4, 5): *1* noradrenaline, *2* adrenaline, *3* DHBA, *4* dopamine

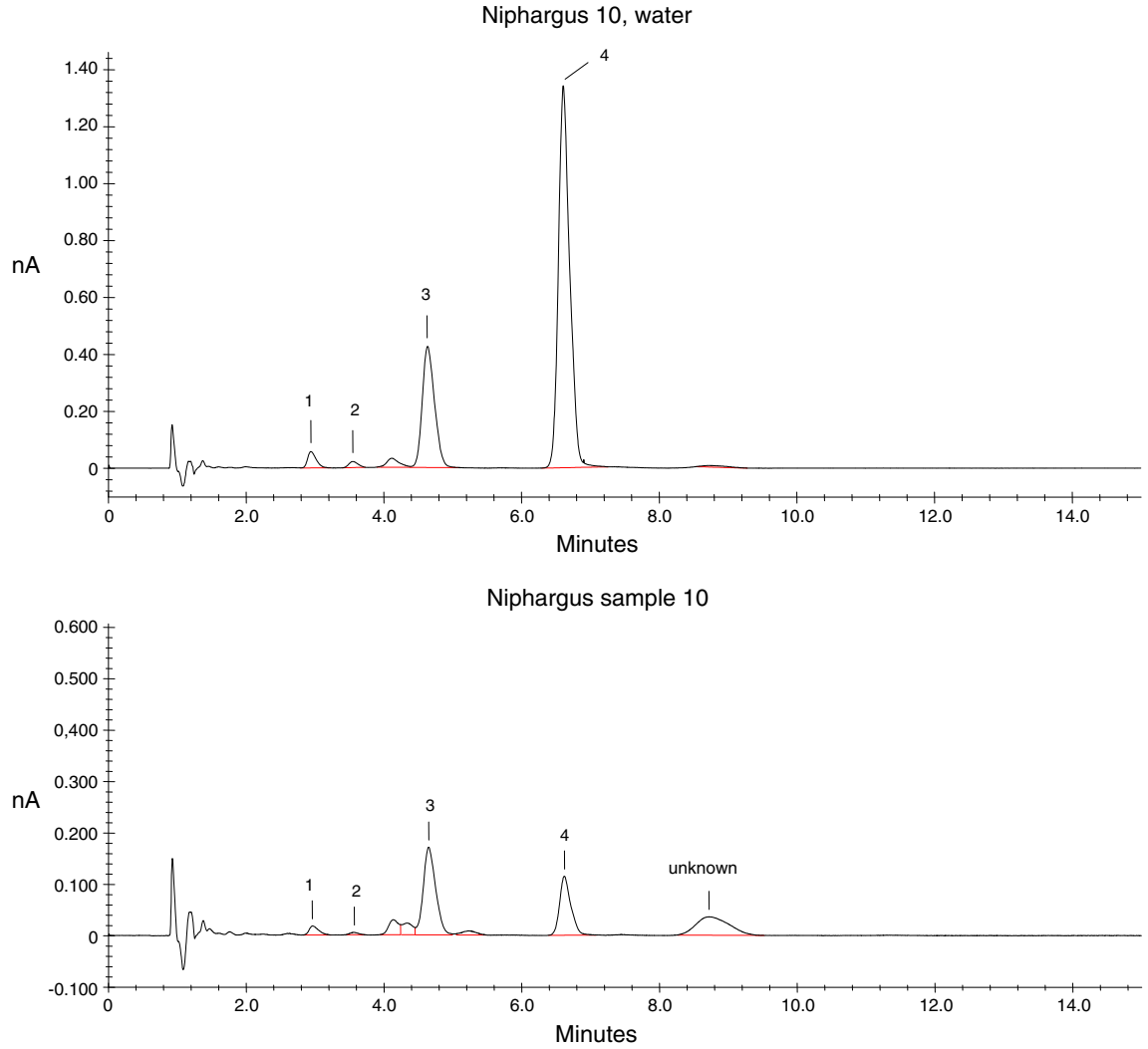

Fig. 4 Chromatograms of catecholamines in a single individual of *Niphargus* (sample 10) and those in the jointly frozen groundwater analyzed separately ("water"). *1* Noradrenaline, *2* adrenaline, *3* DHBA, *4* dopamine

detection limit. Contamination of the sample seems unlikely. During its stress response, a decrease in dopamine level and an increase in adrenaline concentration have been observed in *Chlamys farreri* [2]. Thus, a stressed individual might reveal such CA patterns. This could also be the case for some of the individuals that were observed to swim up to the water surface just before shock-freezing in liquid nitrogen (the samples labeled with [b] in Table 1). We assume that these animals might have suffered some disturbance through unintended slight agitations while the microtube was transferred into the liquid nitrogen.

The samples can be arbitrarily classified into two groups: one with a DA level of more than 14.3 ng/mg, and one with a DA level of below 14.3 ng/mg. This might be an indication that there are different species present among the samples, or that the level of catecholamines is dependent on the life stage and/or size of the individual.

Summing up, the CA levels in the analyzed animals were generally quite high for such small organisms. For example, the CA levels in the heart tissue of whiteleg shrimp *Litopenaeus vannamei* were only about 24 pg/mg fresh weight for DA, 36 pg/mg for NA, and 18 pg/mg for A [12]. These high levels might constitute an adaptation to the harsh living conditions in groundwater. Living and reproducing in groundwater habitats require a high degree of adaptation in true groundwater organisms (stygobionts). Over the course of evolution, these animals have lost their eyes and pigmentation as a consequence of the permanent darkness. Due to the poor availability of food resources and oxygen, stygobionts must frequently cope with starvation and hypoxia. This fact, together with the comparatively constant low temperature, is the reason for their low rates of metabolism and reproduction, and thus their long life spans [21, 37–39]. It can be assumed that, during the course

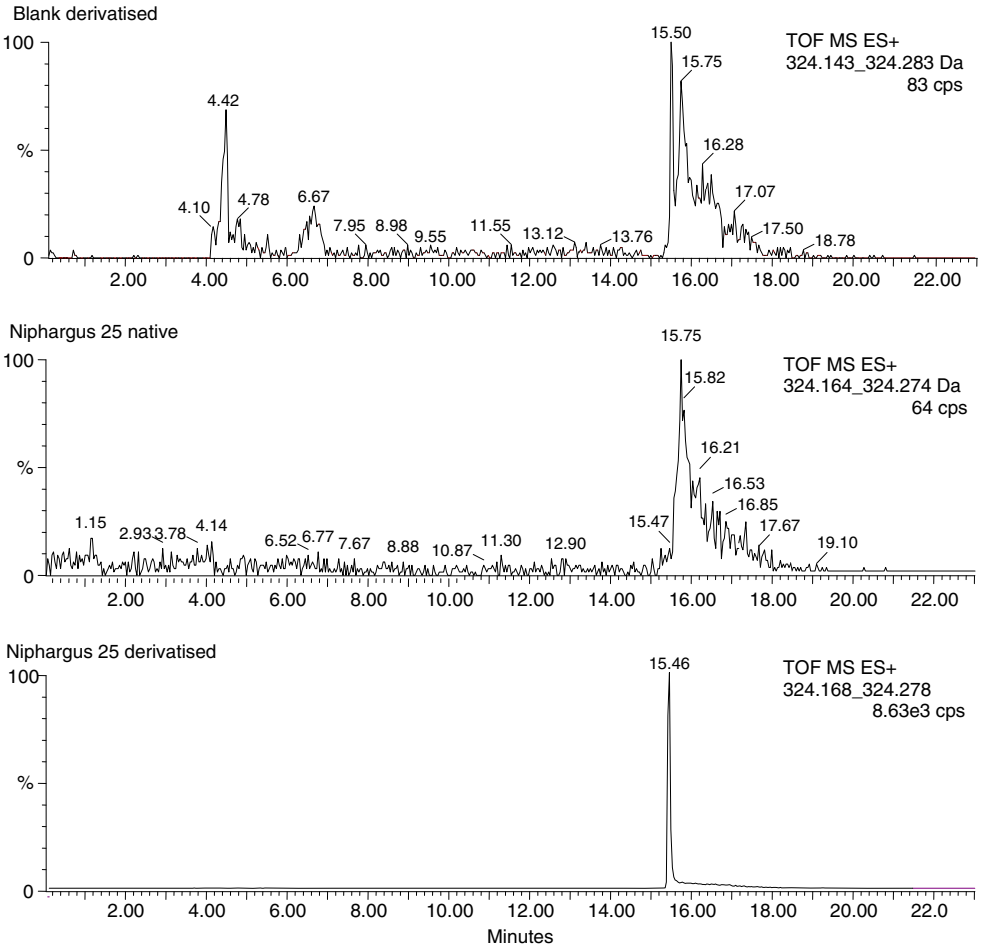

Fig. 5 Mass chromatogram traces at around *m*/*z* 324.2 (theoretical *m*/*z* 324.135 for the AccQ derivative of DA) isolated from TICs of the extract from *Niphargus* sample 25. *From top to bottom*: derivatized blank, native, and derivatized samples

of adaptation, there have also been changes to their stress tolerance mechanisms and—assuming that catecholamines are involved in stress reactions with groundwater amphipods—in catecholamine levels.

Alternatively, shock-freezing in liquid nitrogen (which takes 10–15 s to fully freeze the liquid in the vial) may have been too slow to prevent a panic reaction in the animals. Future experiments with the systematic application of a stress factor will shed more light on this issue.

Experiments to additionally verify the identities of the CAs in *Niphargus* sp. by derivatization and (qualitative) determination in UPLC/TOF-MS

The identification of catecholamines (CA) in *Niphargus* samples via HPLC/EcD analysis was based on a comparison of the retention times of the obtained peaks with those of external standards. Therefore, a more specific additional verification of the chemical identities of the measured compounds was considered to be necessary. Given the very limited sample volumes, mass determination by UPLC/TOF-MS was our preferred method. However, previous experience had shown that, due to the hydrophilicity of the target compounds (noradrenaline, NA; adrenaline, A; dopamine, DA), their chromatographic behavior in eluent systems that are suitable for electrospray ionization (ESI) is not satisfactory (due to a short RT). Together with the chemical lability of the CAs, this caused low sensitivity and reproducibility in MS, even at much higher CA concentrations than found in our samples. Therefore, UPLC/TOF-MS measurements of aliquots of *Niphargus* samples would not allow the direct identification of CAs by mass determination here.

To overcome these problems, aliquots of the extract solutions from *Niphargus* samples 24, 25, 26, and 27 were taken immediately after preparation and derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Waters AccQ reagent kit). This reagent has already been applied for the derivatization of amines [40] and catecholamines [41, 42] in biological matrices using UV absorbance or fluorescence as the usual detection method. We wanted to utilize the advantageous properties of AccQ derivatives of CAs for additional identification by UPLC/TOF-MS. Improved chromatographic behavior and higher ESI-MS sensitivity were expected due to the increased hydrophobicity,

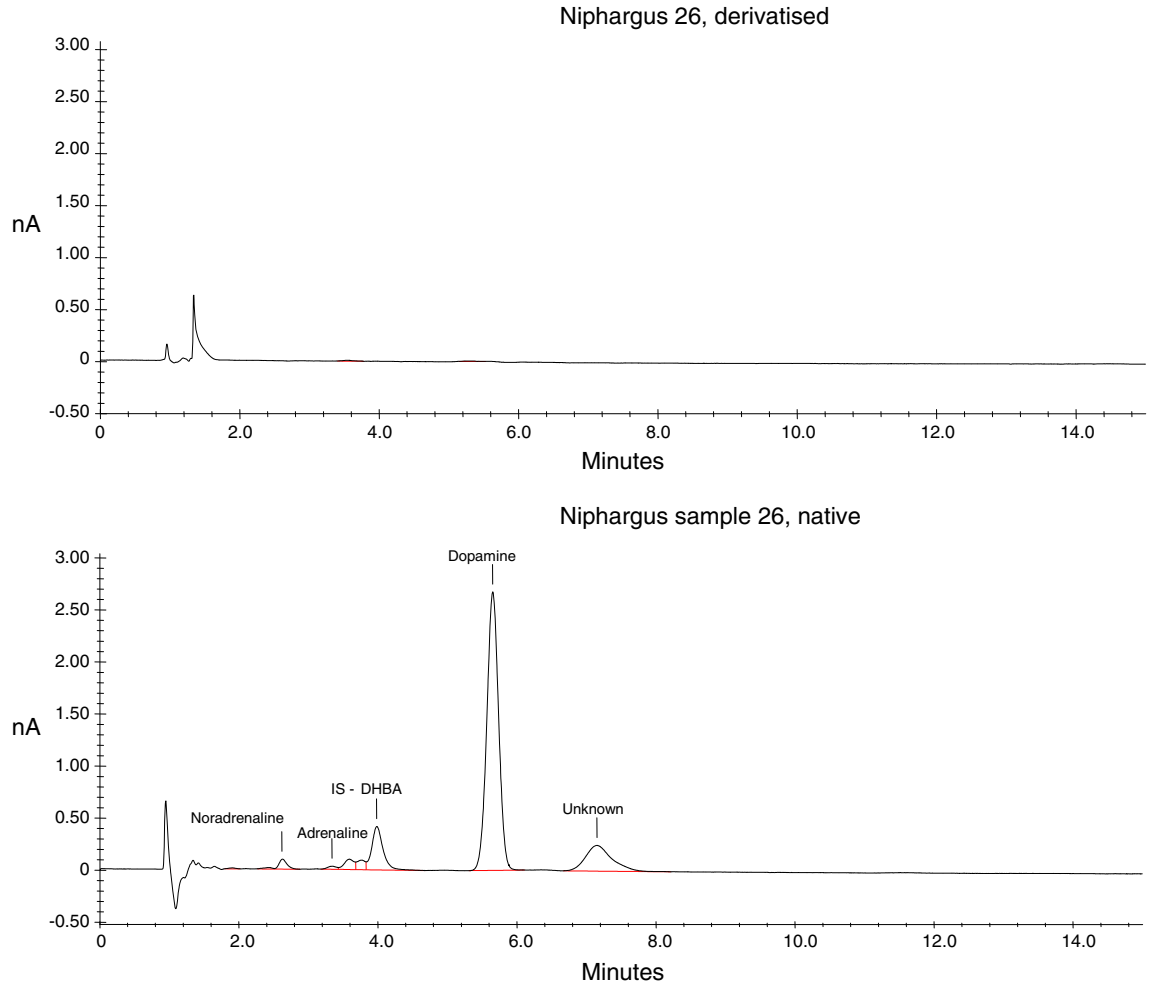

Fig. 6 HPLC/EcD chromatograms of *Niphargus* sample 26: native extract (*bottom*) and extract after derivatization with AccQ•Tag (*top*)

enhanced chemical stability, and considerably higher molecular weights of the derivatives.

In both nonderivatized and derivatized aliquots of the samples, no traces of the native target compounds nor masses calculated for the derivatization products of NA and A could be identified in the corresponding extracted mass chromatograms. However, an intense mass peak at *m*/*z* 324.197±0.06 (MH^+, theoretical: 324.135) at an RT of around 15.5 min confirmed the presence of the DA-AccQ derivative in all of the derivatized sample aliquots. The absence of any other peaks from CAs can be explained by their very low concentrations in the samples compared to DA (an example can be seen in Fig. 5).

Native and derivatized aliquots of the final extract were analyzed as described above in a blank sample that had been extracted in parallel with *Niphargus* samples 25 and 26. As expected, none of the analytes monitored could be detected in these aliquots with our methods (see Fig. 6).

To get an indication of the effectiveness of the derivatization procedure, the derivatized aliquot of *Niphargus* sample 26 was also analyzed with HPLC/EcD. In the resulting chromatogram, the CA peaks had completely disappeared at their characteristic retention times (see Fig. 6), thus clearly indicating a quantitative reaction.

The results of the investigations presented above confirm that the intense peak seen in HPLC/EcD chromatograms of most of the analyzed *Niphargus* samples at the RT of DA can definitely be attributed to this biomolecule.

Improvements to this derivatization method, better adaption to the other CAs and establishment of quantitative measurement of the derivatives with UPLC/TOF-MS were not possible during this investigation, but could be worth future research effort.

Conclusions and outlook

Using two independent analytical methods, we demonstrated that groundwater invertebrates of the genus *Niphargus* do have catecholamines (CAs). Moreover, the levels of CAs

were surprisingly high. This leads us to initially speculate about a close link between CAs and evolutionary adaptation to stable but harsh living conditions. Furthermore, the presence of CAs constitutes the basis for future research on stress response in groundwater invertebrates. If CAs are involved in direct or indirect stress reactions, the potential to develop a stress-response approach for the assessment of groundwater ecosystem status and water quality based on groundwater invertebrates would appear to be good. Without doubt, further studies are needed to evaluate whether catecholamines are involved in the stress response of these animals and, if so, whether CAs are sensitive biomarkers. Additional samples must be analyzed for CAs, and precise measurements of the animals' body weights and sizes must be made in order to evaluate whether the large variations observed are related to differences in the ontogenetic phases of the organisms, the presence of different species, or to variations in stress sensitivity. Moreover, an investigation of the changes in CA concentrations with various types of stress is now necessary as a consequence of our current findings. Nevertheless, in proving the presence of CAs in representatives of the genus *Niphargus*, our study opens the door to a new set of potential stress biomarkers for groundwater organisms. Additionally, the data provide a methodological basis for future studies from an analytical point of view. We propose that the amount of internal standard used should be increased and smaller sample volumes injected so that more accurate quantifications of the surprisingly high dopamine concentrations can be performed. In order to avoid possible losses of CAs during sample analysis, the animals should always be analyzed together with the water in the frozen sample.

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Publication IV

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Catecholamine Levels in Groundwater and Stream Amphipods and Their Response to Temperature Stress

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Abstract

Temperature stress in invertebrates is known to be reflected by changes in catecholamine levels. However, the mechanisms of stress response are not fully understood. Groundwater and surface water amphipods are expected to be differently adapted to temperature elevations due to the different temperature regimes in their habitats and consequently, show a different stress response. No data have been published so far regarding the effects of stress on catecholamine patterns in groundwater invertebrates and accordingly, comparisons with surface water fauna are also missing. In this study, we compared the average catecholamine levels in two taxonomically related amphipod species: *Niphargus inopinatus*, living in groundwater with constant water temperatures throughout the year, and *Gammarus pulex*, a surface water stream amphipod frequently exposed to diurnal and seasonal temperature fluctuations. Furthermore, we tracked the immediate changes in whole-animal catecholamine levels in response to heat stress in both species. Pronounced differences in the catecholamine levels of the two species became apparent, with the average dopamine (DA) level of *N. inopinatus* being almost 1000 times higher than that in *G. pulex*. The noradrenaline

(NA) concentrations in *N. inopinatus* were on average two orders of magnitude higher than in *G. pulex*, and for adrenaline (A), the difference constituted one order of magnitude. When exposed to short-term heat stress, both species showed a response in terms of catecholamine levels, but the observed patterns were different. In *N. inopinatus*, temperature stress was reflected by the appearance of adrenaline, while in *G. pulex* a significant increase in noradrenaline levels occurred in the treatment with the highest temperature elevation.

Keywords: biogenic amine, dopamine, norepinephrine, epinephrine, *Niphargus*, *Gammarus*

1. Introduction

Groundwater invertebrates differ from the invertebrates dwelling in surface water habitats in many physiological aspects. The differences result from various adaptations which the organisms have developed in order to match their specific habitat conditions. Having to deal with food scarcity, comparably low water temperatures, and temporary hypoxia, groundwater invertebrates are known to have (among other adaptations) a reduced metabolism and a much longer life-span than related surface water species (Schminke, 1997; Simčič et al., 2005; Spicer, 1998). It seems conceivable that such physiological differences might also be present in terms of temperature adaptation mechanisms, thus reflecting the different temperature regimes in both habitats. In temperate regions, amphipods living in surface water streams are exposed to strong seasonal, as well as diurnal temperature fluctuations. In contrast, groundwater amphipods experience relatively stable water temperatures throughout the year (the average annual groundwater temperatures in shallow aquifers in Germany ranges from 10 to 12°C (Bannick *et al.*, 2008)). If the differences in temperature conditions did lead to different adaptations, this might also have affected the physiological stress response to heat stress. Following this line of reasoning, it can be assumed that groundwater amphipods should react more sensitively to temperature fluctuations than surface water amphipods.

At present, groundwater organisms are exposed to both relatively slow, long-term regional temperature elevations (due to climate change), as well as fast, local shifts in groundwater temperature resulting from the use of shallow geothermal energy. In Germany, temperature changes of ± 6°C as compared to the mean natural temperatures are legally considered acceptable (Haehnlein et al., 2010). Nevertheless, if groundwater temperature changes are fast and do not allow adaptation, they might influence community structure, activity and diversity (as observed by Brielmann *et al.*, 2009; 2011) and thus may affect the functioning of the entire ecosystem.

Catecholamines (CAs), *i.e.* noradrenaline, adrenaline, and dopamine, are involved in the physiological response to stress and this has been shown not only for vertebrates, but also for a number of invertebrate species, *e.g.* the oyster *Crassostrea gigas* (Lacoste *et al.*, 2001a; Lacoste *et al.*, 2001b), the scallop *Chlamys farreri* (Chen *et al.*, 2008), the insect *Drosophila virilis* (Hirashima et al., 2000), and the whiteleg shrimp *Litopenaeus vannamei* (Aparicio-Simón *et al.*, 2010). Therefore, we assumed that CAs might also be involved in the stress response caused by temperature elevations in freshwater amphipods. The mechanisms mediating the stress response and the physiological functions of CAs in invertebrates are still not fully understood (Aparicio-Simón *et al.*, 2010; Lacoste *et al.*, 2001a). Even though there seems to be an ancestral type of stress response with the basic mechanisms and molecules being well preserved throughout evolution (Ottaviani and Franceschi, 1996), the way CAs act can be species-specific and may differ within different crustacean taxa (Tierney *et al.*, 2003). For example, in the giant tiger prawn *Penaeus monodon*, dopamine (DA) has been shown to induce a release of crustacean hyperglycemic hormone (CHH) to the hemolymph, leading to hyperglycemia (a typical stress response in crustaceans), whereas in the crayfish *Procambarus clarkii*, DA led to the opposite effect via an inhibition of CHH release and subsequent hypoglycemia (Kuo *et al.*, 1995; Sarojini *et al.*, 1995).

Regarding true groundwater fauna (stygobites), the functions of CAs have not been studied so far. To our knowledge, even the presence of CAs in stygobitic crustaceans has been demonstrated only recently, for the amphipod *Niphargus inopinatus* (Pfister et al., 2013). Moreover, there are no direct comparisons available

between closely related surface water and groundwater species in terms of temperature stress response and CAs.

Thus, the objectives of this study were (i) to compare the CA levels in two species of aquatic amphipods from different habitats: *Niphargus inopinatus* (groundwater), and *Gammarus pulex* (surface water), and (ii) to assess the immediate changes in overall CA levels and CA ratios in response to sudden temperature elevations, thereby gaining further insights into the physiological differences in temperature stress response of groundwater and surface water crustaceans. Assuming that *N. inopinatus* would react more sensitively to short-term temperature stress than *G. pulex*, we hypothesized that there would be a pronounced difference in CA levels of stressed and non-stressed individuals in *N. inopinatus*, while in *G. pulex* the differences would be smaller or even negligible.

2. Materials and methods

2.1. Study organisms

Both amphipod species used in the present study were collected from their natural habitats during spring 2011 and 2012. For transport, the animals were transferred into plastic vials filled with ambient water and kept in a cooling box. All animals were acclimated at 12°C and in darkness for at least one week before the temperature stress experiment took place. The water was renewed regularly.

Gammarus pulex LINNAEUS 1758

The gammarids were collected from the river Isar in Munich (Germany). In the laboratory, the amphipods were kept in aerated river water, and fed with pieces of naturally colonized alder and willow leaves from their habitat. Only adult males were used for the experiment in order to keep variability in basal CA levels as low as possible. As most of the animals had formed precopula pairs, each pair was kept in a separate Petri dish until separation of the partners occurred. The males were then transferred into new Petri dishes and kept there until the start of the experiment.

Niphargus inopinatus SCHELLENBERG 1932

The niphargids were collected from groundwater monitoring wells that reach into a shallow Quaternary porous aquifer and are located at the campus of the Helmholtz Zentrum München. Sampling was done using a phreatobiological net-sampler (mesh size: 74 µm; as described in Hahn and Fuchs, 2009). In the laboratory, each specimen was transferred into a separate well within 6-well plates, filled with ambient groundwater and a small amount of sediment and detritus obtained along with the animals during sampling. As there is no possibility to distinguish sexes on live animals without causing significant disturbance, and since in contrast to the gammarids no precopula pairs were observed, both sexes were used for the experiment. However, we tried to reduce variability in basal CA levels by using only niphargids of a defined size range from 3.0 to 4.5 mm, thus presumably obtaining animals of a similar life stage.

2.2. Live determination of body length

As amphipods have a naturally curved body posture, accurate measurements of body length require that the animals be straightened out. Thus, length measurements are usually performed on dead specimens. However, CAs are oxidized quickly at room temperature and therefore, the handling time of the animals had to be kept at a minimum during our experiment. Consequently, determination of body length was performed before the start of the experiment (with the animals still being alive), rather than after the temperature treatment. For this purpose, each animal was photographed alive in lateral position under standardized conditions using a Moticam 2000 digital camera (Motic, Germany). In order to exclude bias resulting from possibly occurring stress, photography was performed some days ahead of the experiment, leaving enough time for recovery of the amphipods. The images were analyzed with the free open-source software ImageJ (version 1.44p). The body length was measured digitally on the photographs with a segmented line along the curved back of the animal, from the base of the first antenna to the base of the telson. The length of the line in pixels was converted to millimeters by means of an appropriate calibration factor. Calibration of the software was done using photographs of calibration slides that

were taken under the same magnification and with the same resolution as the pictures of the respective amphipod species. As body length in living animals varies with the extent to which the body is bent, an average body length was calculated from the analysis of four pictures per amphipod.

2.3. Length-weight regressions for the calculation of dry weight

Dry weight was calculated from body length using length-weight regressions. For *G. pulex*, the equation after Holt and Warrington (1996) was used: $DW = 0.199 + 0.024 * BL + 0.047 * BL^2$

where "DW" is the dry weight [mg], and "BL" is the body length [mm]. The body lengths of the gammarids in the experiment ranged from 10.9 to 20.5 mm, thus corresponding to dry weights ranging from 6.1 to 20.4 mg.

For *N. inopinatus*, a new length-weight regression had to be established as, to the present knowledge of the authors, none was available in the literature. For this purpose, thirty niphargids were photographed alive as described above. Following this, each individual was dried in a drying oven for at least 48 hours, transferred into a pre-weighed tin cup (on average 33 mg) and weighed again (to the nearest 0.002 mg) on a Sartorius CP2P microbalance (Sartorius, Germany). Weight measurements were done in triplicate and the arithmetic mean was used for the regression. Mean length was plotted versus mean dry weight and a non-linear regression of the type $DW = a * BL^b$ was fitted using the 'nls2'-package in R (Grothendieck, 2012). The 'nls2'-package estimates the parameters of a non-linear model by means of the least squares approach using a Gauss-Newton algorithm. The relationship between body length and dry weight was tested for statistical significance by means of a correlation analysis. For this purpose, the Pearson product moment coefficient was computed. Due to ties in the dataset the data were ranked before the calculation. The body lengths of the niphargids used in the temperature-stress experiment ranged from 3.0 to 4.5 mm, corresponding to a dry weight of 0.11 to 0.30 mg, respectively.

2.4. Temperature treatment

In order to simulate a fast, short-term temperature elevation (within a time scale of seconds), two different temperature treatments were performed: A) a sudden rise

in temperature by 6°C, resulting in a change from 12 to 18°C, and B) a sudden rise in temperature by 12°C, leading to a temperature elevation from 12 to 24°C. These temperatures were chosen in accordance with the fact that in Germany, the recommended groundwater temperature thresholds for open geothermal systems allow for a maximum temperature change of ± 6°C, the recommended maximum injection temperature in open-loop systems being 20°C (Haehnlein et al., 2010). The temperature elevations were achieved through the addition of hot water with the necessary temperature into each vial (described in detail below). In a control treatment, water with a temperature of 12°C was added, so that no changes in overall temperature occurred in the vial. Ten amphipods of each species were used in each treatment (except for the 12→24°C treatment with *G. pulex*, where one specimen had to be discarded due to technical problems during CA-analysis). Additionally, in order to estimate the basal levels of CAs in the amphipods, as well as to check for possible mechanical stress resulting from the addition of water into the vials, five individuals of each species were analyzed using the same setup as for the controls, but without the addition of extra water.

2.5. Experimental setup

In order to maintain a stable temperature of 12°C during the acclimation and all experimental procedures, the study took place in a constant temperature room (with maximum temperature fluctuations of ± 1°C, according to the manufacturer's specifications).

The experiment was performed in aluminium cups with a diameter of 60 mm. Each cup was equipped with a hand-made wire bracket that served for positioning a test vial tightly in the centre of the cup. This setup allowed shock-freezing of the animal inside the test vial by pouring liquid nitrogen into the cup. Due to the wire bracket, any disturbance or agitation of the vial during shock-freezing was avoided, thus preventing mechanical stress to the animals.

Falcon™ tubes (15 mL) that were cut down to ¼ of their original length served as test vials. Each vial was filled with 1.4 mL water from the original habitat at a temperature of 12°C and positioned into the centre of the aluminium cup. A single amphipod was transferred into each vial and the vial was then closed with a

rubber stopper. The animals were left to rest and acclimatize in the vials for a minimum of two hours in complete darkness and without disturbance.

Before the resting period, a thick needle was introduced into the rubber stopper of each vial, in a way that its tip exactly reached the water surface, but did not protrude into it. The addition of hot water into the vial was performed through this needle. Another needle was also introduced into the rubber stopper and served as an outlet for excessive air during the water injection. After the resting period, 0.4 mL of hot water were injected through the needle into each vial. For the 12→18°C treatment, water with a temperature of 54°C was used, whereas water with 92°C was added in the 12→24°C treatment. Preliminary experiments had shown that the water mixed immediately, so that no direct exposure of test animals to the hot water occurred. Ten seconds after the temperature rise, each animal was shock frozen by pouring liquid nitrogen into the aluminium cup.

No direct measurements of the water temperatures in the vials were performed during the experiment in order to avoid mechanical disturbance to the animals. However, it was verified in preliminary experiments, as well as with test injections in parallel vials without animal immediately prior to the actual experiment, that the desired temperatures were indeed established in the vials. The intra-vial temperatures that were reached after the water injections in the preliminary experiment were 18.6 ± 0.3°C in the 12→18°C treatment and 24.8 ± 0.6°C in the 12→24°C treatment (average ± standard error of 10 trials per treatment).

During all preparations, the experiment was kept in darkness. The temperature manipulation itself and the subsequent shock-freezing were performed using a dim and indirect light source in order to avoid light disturbance as much as possible.

2.6. Species determination and addition of the antioxidant

As determination of both amphipod species requires microscopic examination, the taxonomic identity of the test animals could be verified only after the end of the experiment. For this purpose, each sample had to be briefly defrosted. In order to prevent oxidation of the CAs during handling and later sample storage, 450 µL of an aqueous sodium metabisulfite solution were added at a final concentration of 2 g L^{-1} immediately after the sample had thawed. The now liquid samples were kept

on ice during microscopy and the total handling time rarely exceeded two minutes per animal. After species determination, the samples were immediately refrozen in liquid nitrogen and subsequently stored at -70°C until further processing.
The microscopic examination revealed that 5 specimens in the 12→18°C treatment, 2 specimens in the 12→24°C treatment and one specimen in the controls represented *Gammarus fossarum*, another gammarid species occurring together with *G. pulex* in the Isar river. These eight animals were excluded from the dataset and instead, 8 additional individuals of *G. pulex* were collected during the same time of the following year (spring 2012), and subjected to the same experimental procedures as described above. The CA data of *G. fossarum* were only included in the broader comparison with literature data (see discussion section).

2.7. Extraction and analysis of catecholamines

Due to the small size of the niphargids (average body length: 3.8 mm), the CAs in both amphipod species were extracted from the whole animal rather than from specific parts of the nervous system or the hemolymph. The analysis of CAs was done using a commercial kit for the extraction of CAs from human blood plasma (ClinRep® Complete kit for catecholamines in plasma, RECIPE, Germany), followed by a quantitative analysis of noradrenaline (NA), adrenaline (A), and dopamine (DA) by reversed phase HPLC coupled to an electrochemical detector. The procedures of the kit were adjusted to suit the individual requirements of CA analysis in the two different amphipod species (described in detail below). Test measurements of aliquots of a sample of pooled specimen of *N. inopinatus* revealed that the resulting extraction method and the subsequent analysis at the HPLC/ EcD had a relative uncertainty of 15.2% for NA, 17.3% for A, and 9.9% for DA (corresponding to the standard deviation divided by the average). The accuracy of the mean (defined as the observed deviation from an expected value in %) was found to be 9.6% for NA, 13.4% for A, and 2.6% for DA (Pfister et al., 2013), tested with a CA standard (800 pg/ sample in 1M HCl, Thermo Scientific). The limit of detection was found to be 0.33 pg/ µL for NA, 0.2 pg/ µL for A, 0.33 pg/ µL for DA and 0.2 pg/ µL for the internal standard dihydroxybenzylamine (DHBA).

CA extraction in *Gammarus pulex*

The extractions of CAs from the amphipod and from the water in each sample were performed separately. After thawing the sample at room temperature, the animal was transferred into a polypropylene vial containing 0.7 mL 2 M Tris buffer (RECIPE®), 200 µL of a 10 pg/ µL DHBA solution as internal standard (RECIPE®) and 100 µL of a 5 g/ L sodium metabisulfite solution in Tris buffer. The mixture was homogenized on ice by an Ultra-Turrax T25 homogenizer (8 mm tool, IKA Labortechnik, Germany) for 5 sec at 24000 rpm (suspension A). 300 µL Tris buffer were used to clean the Ultra Turrax from the remaining animal tissue, performing a short run (1 sec) at 8000 rpm (suspension B). In a second homogenization step, suspension A was treated with an ultrasonic homogenizer for 40 sec (micro-Sonotrode tip, Bandelin Electronics) while ice-cooled. For rinsing the sonicator tip, 200 µL Tris buffer from suspension B were used. Subsequently, both suspensions A and B were centrifuged for 2 min at 1800 g. For removal of still remaining chitin particles, the combined supernatants were filtered (Acrodisc® 25 mm syringe filter, with 1 µm glass fiber membrane) into the alumina adsorption column delivered with the CA extraction kit (sample preparation column, RECIPE® ClinRep®), but supplemented with an additional 8 mg of alumina adsorbent (RECIPE®).

The water sample was transferred into a self-packed 5 mL Pierce® centrifuge column (Thermo Scientific, U.S.A.) with two 10 µm polyethylene frits (Isolute® SPE Accessories, Biotage), and containing 30 mg alumina, 0.5 mL Tris buffer and 200 µL DHBA (10 pg/ µL) as internal standard. The column was incubated for 10-12 min (during the animal homogenization steps) at room temperature. In the next step, both adsorption columns were vortexed for 15 min to sorb the CAs onto the alumina (as in Goldstein *et al.*, 1981). After the adsorption, the alumina was washed three times by repeatedly adding 1 mL washing solution (RECIPE®), shaking the columns for 10 sec, and aspirating the supernatant. Subsequently, the CAs were eluted by adding 160 µL elution reagent (RECIPE®) to the column. The column was then vortexed for 5 min and the obtained eluate was centrifuged for 2 min at 600 g. The eluate was then transferred into an HPLC vial and an aliquot of 40 µL was injected into the HPLC system. Three separate injections were performed for each sample.

CA extraction in *Niphargus inopinatus*

The extraction of CAs from *N. inopinatus* was performed in the same way as described for *G. pulex*, except for the homogenization step. Here, the samples were only homogenized by means of ultrasonication (40 sec). Additional grinding with the Ultra Turrax was not necessary due to the much smaller size and fragility of the niphargids. Subsequently, the samples were centrifuged and supernatants were directly transferred into the adsorption column (RECIPE®).

CA analysis by HPLC/ EcD

Identification and quantification of the extracted CAs was performed by reversed phase HPLC (with a 150 x 4.6 mm column for catecholamines in plasma, RECIPE®) coupled to an electrochemical detector (Dionex Corporation, USA). The detector potential was set to +700 mV and the flow rate of the degassed mobile phase (included in the CA-extraction kit, RECIPE®) was 1 mL/ min. The identification of CAs was carried out by comparing the retention times (RT) of the peaks from the samples to the known RT of a previously analyzed mixture of calibration standards. For CA quantification, the ratio of the peak area recorded for a certain CA to the peak area of the internal standard in the sample was compared to the corresponding ratios obtained from samples with known concentrations of calibration standards. Only peaks with a signal-to-noise ratio greater than 3 were evaluated.

2.8. Statistical analyses

Normal distribution of the data was tested with a Shapiro-Wilks-Test in each data subset and normality was assumed at p-values above 0.2. Due to the small sample size of the subsets, graphical methods were additionally used to verify the result (*i.e.* boxplots, histograms, as well as Q-Q-plots). As most of the CA-data in the different treatments were not normally distributed, a Kruskal-Wallis rank sum test was applied to test for (i) interspecific differences in CA concentrations (using only the basal CA-levels of the undisturbed specimens from the treatment without water injection), and (ii) for significant effects of temperature elevation within each of the two species (using the temperature-treated specimens and the controls

with water injection). In case of significant test results, *post hoc* comparisons were done using two-sided Wilcoxon rank sum tests. A Bonferroni correction was applied to account for multiple comparisons, resulting in a corrected significance level $\alpha_{corr} = \alpha/k$, with 'k' being the number of comparisons performed and 'α' being the commonly used significance level of 0.05. Differences between basal CA amounts and the CA concentrations in the control treatments were also tested using the Wilcoxon test. In some data subsets, a variance of zero occurred since no CAs were detected in any of the samples (*i.e.* for adrenaline in the undisturbed niphargids, as well as in the controls). Here, a qualitative comparison was performed with the other subsets, as statistical testing was not appropriate. All statistical analyses were performed in the software environment for statistical computing R (R Development Core Team, 2010).

2.9. Unit conversions and underlying assumptions

In order to compare the results from this study to the data reported in other publications (see discussion section), some unit conversions were performed, so that all data in the figure are now presented in 'pg/ mg fresh weight'. The data in Ehrenström and Berglind (1988) for *Daphnia magna* were originally given in 'ng/ mg protein'. These values were roughly converted into 'pg/ mg fresh weight' under the assumption that the amount of CAs present in 1g of wet tissue corresponds to the amount present in 16.9 mg of protein. This assumption was based on information from the original publication, stating that the average basal levels of DA in *Daphnia magna* of 64.92 ng DA/ g wet tissue corresponded to 3.837 ng DA/ mg protein (in undisturbed animals). The data from the present study (originally in 'pg/ mg dry weight') were converted into 'pg/ mg fresh weight', based on the assumption that the dry weight of an amphipod corresponds on average to 23% of its fresh weight. This number has been determined for *Gammarus pulex* by Aschauer *et al.* (2010).

3. Results

The interspecific comparison of whole-animal CA levels showed large, statistically significant differences ($p<0.05$) between *N. inopinatus* and *G. pulex* with respect to NA and DA. The average whole-animal DA content in *N. inopinatus* was almost

1000 times higher than in *G. pulex* (basal CA levels from the undisturbed treatment without water injection; Table 1). Also, the average NA concentrations were 200 times higher in the niphargids as compared to the gammarids. For adrenaline, the differences were not as pronounced, but in contrast to *G. pulex*, where a low amount could be detected even in the 'undisturbed' specimens, in *N. inopinatus* no adrenaline was found. The average CA-ratios were also much higher in *N. inopinatus* than in *G. pulex* (Table 2). These marked interspecific differences were similarly observed also in the heat-treated animals (Fig. 1).

Table 1: Basal catecholamine levels (n = 5 for each of the two species). All concentrations are given in pg/ mg dry weight.

N. inopinatus			
	Dopamine	Noradrenaline	Adrenaline
Mean (± s.d.)	**67971.1 (±21625.7)**	**2042.5 (± 875.3)**	**0**
Minimum	47866.9	1496.0	0
Maximum	101812.8	3575.3	0
G. pulex			
	Dopamine	Noradrenaline	Adrenaline
Mean (± s.d.)	**68.2 (± 44.4)**	**9.8 (± 17.0)**	**6.5 (± 6.1)**
Minimum	38.9	0	0
Maximum	140.2	39.7	14.3

Table 2: Average catecholamine ratios in *N. inopinatus* and *G. pulex* as related to different short-term temperature elevations. *Due to lack of adrenaline, the NA:A-ratio could not be computed in the control treatment for *N. inopinatus*.

	N. inopinatus				***G. pulex***			
	control	12→18°C	12→24°C	pooled data	control	12→18°C	12→24°C	pooled data
DA:NA	39.9	25.1	44.2	36.0	21.7	13.9	1.2	4.4
NA:A	*	13.6	7.7	16.7	0.2	0.5	1.7	0.9

The sudden temperature elevations resulted in a statistically significant effect (p=0.003, Kruskal-Wallis test) on whole-tissue NA concentrations in *G. pulex* (white bars, Fig.1). Thus, in the treatment with the highest temperature elevation, the average NA concentration was significantly higher than in the control treatment (p=0.001, Wilcoxon test). Moreover, the average DA:NA-ratio decreased with increasing temperature elevations, while the NA:A-ratio increased (Table 2).

In *N. inopinatus*, the temperature treatments also induced a measurable response in catecholamine levels. Thus, A could be detected exclusively in the treatments with elevated temperature, while in the controls, no A was present (black bars, Fig. 1). This resulted in a variance of zero in the controls, so that the temperature effects on A could not be tested statistically. Nevertheless, the average concentrations of A showed an increasing trend with increasing temperature. In contrast, the average NA concentrations showed an opposite (though not statistically significant) tendency, thus decreasing with increasing temperature. Regarding CA ratios, no consistent response in relation to temperature treatments was observed (Table 2).

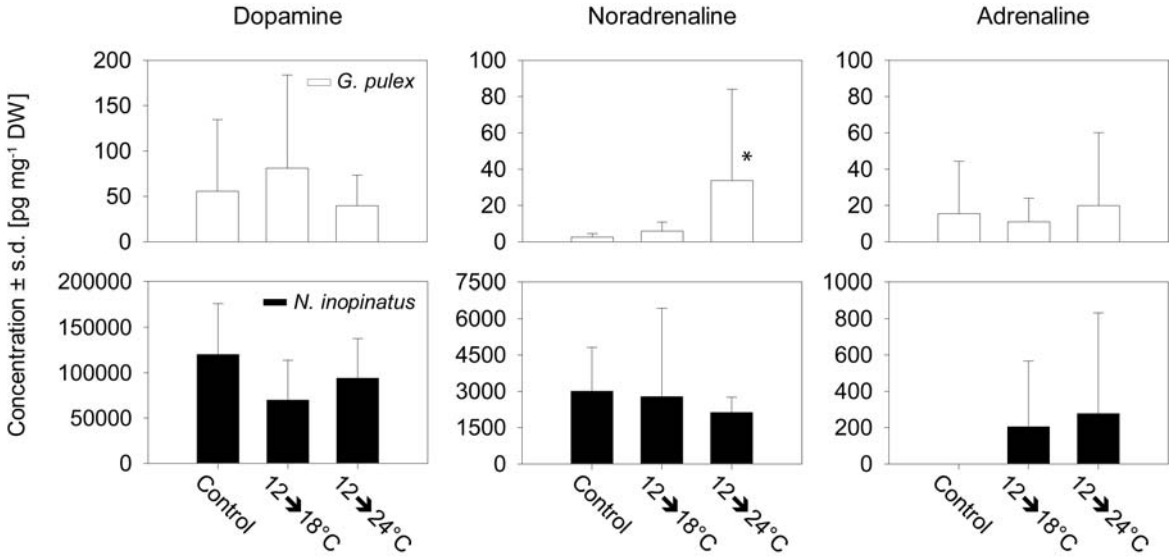

Figure 1: Catecholamine levels in the surface water amphipod *Gammarus pulex* and the groundwater amphipod *Niphargus inopinatus* after a sudden temperature elevation. The NA levels in *G. pulex* marked with an asterisk were significantly different from the control. Sample size was n = 10 for each treatment and each species, except for *G. pulex* in the 12→24°C treatment, where n = 9 due to technical problems during CA analysis in one of the samples. Note the different Y-axes.

The DA levels in the control treatment of the experiment differed significantly from the basal levels determined for *N. inopinatus* (p=0.04, Wilcoxon test). Thus, the mechanical disturbance during the addition of water into the experimental vials must have already been sufficient to affect the stygobitic amphipods. For *G. pulex*, no such effects were found.

The relationship between the body length and the dry weight of *N. inopinatus* was highly significant (Pearson's $\varrho = 0.92$, $p < 0.001$). It was described well by the

equation $DW = a * BL^b$, with parameters a = 0.0062 and b = 2.5837 (R^2 = 0.91, Fig. 2). The 95% confidence intervals for the parameters '*a*' and '*b*' were [0.0035 – 0.0107] and [2.2537 – 2.9178], respectively.

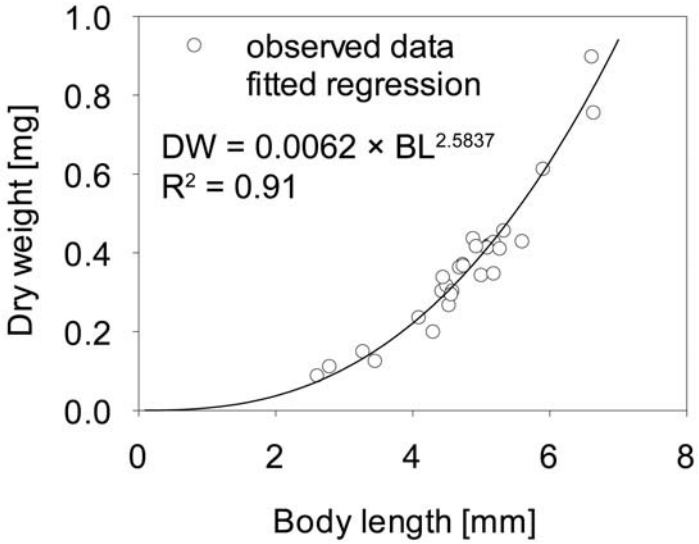

Figure 2: Length-weight regression for *Niphargus inopinatus* (n = 30). The abbreviations used in the regression equation are: 'DW' – dry weight, and 'BL' – body length.

4. Discussion

4.1 Interspecific comparison

For the biosynthesis of CAs, the amino acid L-phenylalanine is required, which is one of the ten essential amino acids that cannot be synthesized by crustaceans and need to be taken up from the ingested food (as reviewed in Boghen and Castell, 1981). Phenylalanine hydroxylation leads to the formation of L-tyrosine, which is then further hydroxylated to L-DOPA by *tyrosine-hydroxylase*. This second hydroxylation is the rate-limiting step in CA biosynthesis, with L-DOPA being the direct precursor for DA. Subsequently, NA is formed from DA (through another hydroxylation step), and A is formed from NA (via methylation).

The concentrations of all three CAs were significantly higher in *N. inopinatus* than in *G. pulex,* with the high (almost 1000-fold) difference in average DA levels being particularly striking. Considering that stygobites live in an energy-limited

environment, the question arises, why such high amounts of biogenic amines are present in niphargids. With food being often very scarce in groundwater habitats, stygobites cannot rely on a regular food supply and thus, efficient storage and utilization of CAs (or their precursors) might be essential for survival (Jeffery, W. R., pers. comm.). Furthermore, the explanation for the observed differences can be sought in the physiological functions of the CAs.

DA modulates motor circuits in response to environmental stimuli in a number of animal phyla, *e.g.* Nematoda, Platyhelminthes, Annelida, Mollusca, Arthropoda, and hence, it is assumed that this could be one of the ancestral functions of DA in the invertebrate nervous system (Barron *et al.*, 2010). In addition, heat stress has been shown to cause an increase in whole-animal body content of DA in *Drosophila virilis* (Hirashima et al., 2000; Rauschenbach et al., 1997), suggesting that DA was involved in heat-related stress response in these organisms. However, as whole-animal extracts do not allow direct conclusions on the concentrations of CAs in the hemolymph and in target organs, we can only speculate on the physiological meaning of the interspecific differences found in our study. Nevertheless, such comparisons can serve as a basis for the formation of new hypotheses for future investigations. For example, DA has been shown to have cardioexcitatory effects in several species of crustaceans (reviewed in Fingerman et al., 1994) and hence possibly prepares the organism for a quick flight response by increasing the heart rate. As stygobitic crustaceans are blind, they cannot rely on visual inputs as a means of early detection of danger and hence might be even more dependent on a quick stress response. Indeed, when a niphargid and a stygobitic isopod once met in a Petri dish in our laboratory, both animals quickly changed their crawling directions as soon as they had sensed each other with their antennae (M. Avramov and C. Griebler, unpublished). Being ready for a rapid flight reaction can be crucial for survival and in this regard, having a large quantity of DA in store might represent an adaptation strategy to groundwater habitats. The presence of high amounts of CAs in storage granules located in the salivary gland has been shown for *G. pulex* (Elofsson *et al.*, 1978). Such granules might also be present in *N. inopinatus*. Furthermore, in adaptation to the frequent and often long-lasting food-scarcity periods in groundwater, this mechanism might be developed to an even greater extent, thus enabling the storage of high amounts of DA and allowing the

organism to circumvent the rate-limiting step in biosynthesis. The fast appearance of A in the heat-stressed niphargids observed in our study supports this hypothesis, and so does the animals' apparent sensitivity to short-term mechanical stress.

Another possible line of argument regarding the observed interspecific differences in CA levels can be sought in the evolutionary model discussed in Lacoste *et al.* (2001a). The model suggests that the adrenergic stress response may have evolved from a NA-based system requiring high CA concentrations to an A-based system requiring lower CA concentrations. This trend is supposed to be driven by an increase in sensitivity of the adrenoreceptors and an evolutionary up-regulation of the enzymatic processes required for A biosynthesis (Lacoste et al., 2001a). In our study, the whole-body DA content of *G. pulex* was in the same range as has been recorded for the cladoceran *Daphnia magna* by Ehrenström and Berglind (1988), while the DA levels of *N. inopinatus* were three orders of magnitude higher (Fig. 3) and were comparable to the ones found in another stygobite, the isopod *Proasellus cavaticus* (K.W. Schramm, unpublished), as well as the fruit fly *Drosophila virilis* (Rauschenbach *et al.*, 1997). The model would suggest that niphargids are residing at a more primitive stage of evolution than gammarids. This view can be supported if we assume that for stygofauna, selection pressure was high during ancient groundwater colonization millions of years ago (Conway Morris, 1995; Humphreys, 2000), but once adapted, it became lower due to the stable environmental conditions. In contrast, surface water amphipods might have been subject to a continuous selection pressure due to frequently occurring changes in environmental conditions and competition with other species.

Another possible explanation for the interspecific differences in DA contents is related to the fact that DA affects the release of hormones that mediate gonadal maturation in crustaceans. As reviewed in Tierney *et al.* (2003), *in vivo* injections of DA have been reported to cause smaller testicular size and fewer mature sperm, as well as smaller and less mature oocytes in the crab *Uca pugilator* and in the crayfish *Procambarus clarkii*. Moreover, female individuals of a mutant line of *D. virilis* with a higher level of DA than the wildtype have been found to be less fertile (as summarized by Hirashima *et al.*, 2000). Stygobitic species are known to reproduce less frequently and to have lower egg numbers as compared to

taxonomically related surface water species, which is considered an adaptation to their energy-limited environment. Thus, it seems conceivable that in groundwater crustaceans, DA might be involved in the slowdown of reproductive functions or in their suppression under unfavorable conditions.

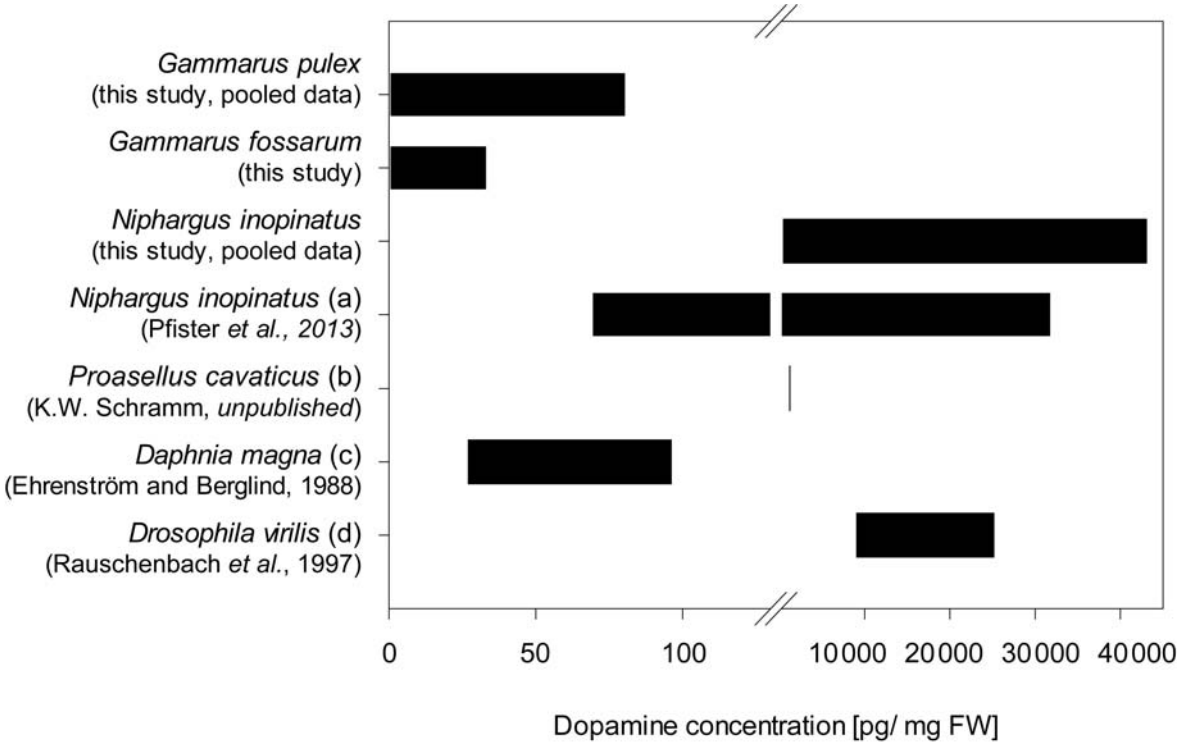

Figure 3: Comparison of dopamine ranges that have been reported for different arthropod species (whole animal extracts). The data from the present study were converted to [pg/ mg FW] for the comparison in this figure; (a): basal dopamine levels in undisturbed animals (Pfister et al., 2003); (b) dopamine level determined from a pooled sample of 14 individuals of the groundwater isopod *Proasellus cavaticus* (K.W. Schramm, unpublished); (c): dopamine range from an experiment dealing with diurnal variations in dopamine levels of animals exposed to different light regimes (data extracted from Ehrenström and Berglind, 1988); (d): dopamine range from a dataset of heat-stressed (60 minutes at 38°C) and non-stressed flies (data extracted from Rauschenbach *et al.*, 1997).

Last but not least, high DA levels have also been suggested to be linked to aggressive behaviour and the ability to fight, *e.g.* in the crab *Carcinus maenas* (Sneddon *et al.*, 2000). Groundwater ecosystems are thought to be less influenced by biotic pressures (*e.g.* competition or predation) than surface waters, due to the low numbers of individuals (Gibert et al., 1994). Accordingly, subterranean life might rarely offer an opportunity for aggressive behaviour. However, in a food-limited environment, the ability to quickly overwhelm rivals and consequently use them as a food source might be a useful strategy for survival. In support of this

assumption, cannibalism has been observed in our laboratory for *N. inopinatus*, thus posing the question whether high DA levels might be involved in the regulation of aggressive/ fighting behaviour in niphargids as well.

4.2 Temperature stress

The two amphipod species compared in this study differ in their long-term temperature tolerance: while *G. pulex* reaches its maximum growth rate between 12 and 20°C (Sutcliffe *et al.*, 1981), a temperature of 20.2°C is already lethal for half of the tested individuals of *N. inopinatus* after 25 days (Brielmann *et al.*, 2011). In the short-term, the thermotolerance of the niphargids is rather comparable to that of newborn gammarids: in the same two studies, none of the tested (adult) individuals of *N. inopinatus* survived longer than 48 hours at a temperature of 27°C, and similarly, newborn individuals of *G. pulex* did not survive more than four days at a temperature of 28°C. Nevertheless, unlike niphargids, the gammarids frequently encounter (and tolerate) temperature fluctuations in their habitat. Therefore, we expected that while a sudden temperature elevation by 6 or even 12°C might cause some stress to both amphipod species in our study, *G. pulex* would still be more tolerant than *N. inopinatus* and show smaller or no effects in terms of CA levels. The latter could not be confirmed by our results, as the temperature treatment did only lead to statistically significant changes in overall CA levels in *G. pulex*; in *N. inopinatus*, a decrease in average NA levels (accompanied by an increase in average A levels) occurred with increasing temperature, but the trend was not statistically significant. However, this outcome should be interpreted with caution, as it is possible that the duration of the treatments did not match ideally the time point at which the effects occurred. When analyzing whole-animal extracts, a change in CA levels can either occur if (i) new CAs are formed from precursor molecules or released from conjugates, if (ii) there is conversion from one biogenic amine into another (*i.e.,* from DA to NA, and from NA to A), or if (iii) CAs are being inactivated or enzymatically degraded. The naturally low abundance of stygofauna in the field (and the resulting low number of specimens available for experimental work) dictated that shock-freezing of the animals was performed at one specific time point of observation, rather than allowing the assessment of a time-course that requires the freezing of

several individuals per time point. Thus, the data in this study represent only a snapshot and do not allow a continuous tracking of CA conversion processes. Nevertheless, preliminary observations on small numbers of amphipods in our laboratory had indicated that whole-animal CA levels and their ratios can change rapidly (<1 minute) in response to mechanical stress in both species (M. Avramov and T. M. Rock, unpublished). Moreover, the occurrence of A in some of the "temperature-stressed" individuals of *N. inopinatus* in contrast to the complete lack of A in the controls, as well as the above-mentioned (even though non-significant) opposite trends of average NA and A concentrations, support the assumption that at least an initial conversion of NA to A was taking place. In *G. pulex*, the short-term temperature elevation was even sufficient to cause a statistically significant increase in NA levels, indicating that whole-animal CA levels can change within seconds under stressful conditions.

5. Conclusions

The whole-body CA contents in *N. inopinatus* were significantly higher than in *G. pulex*. In *G. pulex*, a significant increase in NA levels occurred with a 12°C-rise in water temperature, while in *N. inopinatus* the observed effects were not statistically significant. However, this does not necessarily mean that *G. pulex* was more sensitive towards temperature stress than *N. inopinatus*, but rather suggests the conclusion that stress response was different in each of the two species. In *G. pulex*, a conversion of DA to NA was probably occurring at the time point of sampling, which is indicated by the opposing trend of sinking DA:NA and rising NA:A ratios, as well as by the rise in average NA levels with increasing temperature stress. In *N. inopinatus*, already the next step in CA conversion from NA to A was probably taking place, which could be observed through the fast occurrence of A in the treatments with elevated temperature as opposed to the controls, where no A was found. Therefore it seems conceivable that in niphargids the conversion steps of CAs are being performed faster than in gammarids, which might be explained through an enhanced storage and higher availability of DA in *N. inopinatus*.

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Author contributions

M.A., C.G., and S.I.S. developed the study concept and were primarily involved in data interpretation. M.A. designed the setup, performed the experimental work and wrote the manuscript. G.P., T.M.R., and K.W.S. conducted the CA analysis including method optimization and QA/ QC and should be primarily addressed about this field. In addition they assisted in experimental concept development and contributed to the method section of the paper. All authors were involved in revising and editing of the manuscript.

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List of Abbreviations

A	adrenaline
BTEX	benzene, toluene, ethylbenzene, and *ortho-*, *para-* and *meta-*xylene
CA	catecholamine
CTMax	Critical Thermal Maximum
d	day
DA	dopamine
DOC	dissolved organic carbon
e.g.	*exempli gratia* (Latin) – for example
et al.	*et alii* (Latin) – and others
EU	European Union
Fig.	Figure
FW	fresh weight
GC/ MS	Gas chromatography–mass spectrometry
GWD	the European Commission Groundwater Directive 2006/118/EC
h	hour
HMGU	Helmholtz Zentrum für Gesundheit und Umwelt (German Research Center for Environmental Health)
HPLC/ EcD	high-performance liquid chromatography coupled with an electrochemical detector
i.e.	*id est* (Latin) – that is; in other words
LC_{50}	concentration of a toxicant that is lethal to 50% of the tested organisms

LT_{50}	temperature that is lethal to 50% of the tested organisms
min	minute(s)
NA	noradrenaline
sec	second(s)
SSD	Species Sensitivity Distribution
UN	United Nations
UPLC/ TOF-MS	ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry
VOC	volatile organic compound
WFD	the European Commission Water Framework Directive 2000/60/EC

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