Spring Gammarids in Difficulties?

Gammarus fossarum Exposed to Copper and Rising Temperatures

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Prof. Dr. Jörg Schibler

Dekan

For my parents

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CHAPTER ONE

General Introduction

Global Change: Temperature

Global Change is the term used to describe changes which affect the environment on a global scale (Lexikon der Geowissenschaften, 2000). As the definition states it is a global phenomenon and one of the greatest challenges of our time. The new IPCC 2014 has just been published (November 1st 2014) and is worded strongly, clearly stating how prevalent the ongoing climate change is, having already influenced the earth dramatically (IPCC, 2014). The period of 1983 to 2012 was likely the warmest 30-year period of the last 1400 years in the Northern Hemispherem (IPCC, 2014). Increasing discharge variability with summer droughts and heavy rainfall in winter, rising temperatures and increasing variability of climatic factors in general will affect all terrestrial and aquatic ecosystems. Hot extremes, heat waves and heavy precipitation events are becoming more frequent (IPCC, 2014) and changes in temperature and precipitation are already noticeable (e.g. Della-Marta et al., 2007).

Even Central Europe is affected by extreme events such as long droughts in summer, as was shown by the heat wave in the year 2003 in Switzerland (e.g. Zappa & Kan, 2007). Within the next century a temperature increase of 1.1 to 6.4 °C has been predicted (IPCC, 2007). Since 1970 the mean air temperature has already increased by 1.5 °C in Switzerland (OcCC, 2008). An increase of the mean winter precipitation in the northern and western part of Switzerland and an increase of heavy precipitation events has also been documented for Switzerland (Schmidli et al., 2002; Schmidli & Frei, 2005).

It is still unclear what exact influence Global Change could have on freshwater biodiversity and how freshwater species will respond to these changes (e.g. Heino et al., 2009). It has already been shown that freshwaters and their biodiversity are especially vulnerable towards climate change (Heino et al., 2009; Woodward et al., 2010). Carpenter et al. (1992) expect that the temperature increase will have a great impact on the structure and functioning of freshwater ecosystems. The spectrum of consequences has recently been reviewed by Kernan et al. (2010).

Many species, including freshwater species, have shifted their geographic ranges, seasonal activities, migration patterns, abundances, and species interactions in response to these ongoing changes in climate (IPCC, 2014).

The water temperature is one of the key factors that determine the life cycle characteristics, such as embryonic development and emergence, of invertebrates (e.g. Haidekker & Hering, 2008). In Swiss rivers warming was already recorded during the last quarter of the 20th century (Hari et al., 2006), for example in the Upper Rhone River (Daufresne et al., 2003). In response to Global Change, species are already expanding their ranges to higher latitudes and altitudes (Krajick, 2004; Heino et al., 2009). Extreme weather events can accelerate shifts in species composition and distribution (Jentsch et al., 2007). Cold-stenothermal species especially will experience range shifts or restrictions, as space with suitable thermal conditions decreases (Woodward et al., 2010). It is assumed that some of these species will become extinct and that extinction rates of freshwater species will exceed those assessed for terrestrial species (Heino et al., 2009). However, it is difficult to make such predictions as climate change interacts with other stressors (Durance & Omerod, 2007), for example landscape fragmentation and destruction and environmental pollution, resulting in Global Change. The results of these interactions are complex cause-effect chains since many environmental parameters are linked to precipitation and temperature (Hering et al., 2010).

Ecotoxicology: Copper

Ecotoxicology is the science of the distribution and effects of harmful substances on organisms or ecosystems, provided that damages for nature and humans occur directly or indirectly (Lexikon der Geowissenschaften, 2000; Fent, 2013). Considering the effects which Global Change is having on the environment, ecotoxicologial studies are becoming even more important for protecting many species which are becoming endangered. The species are endangered by the environmental changes and pesticides which are being used, for example, to ensure high yields of crops in spite of the less favourable environmental conditions such as high amounts of precipitation in a very short time span. It has been shown that changes in climate have had negative impacts on crop yields (IPCC, 2014). The contamination of our environment has taken on new levels because of the more frequent

and widely distributed use of chemicals such as pharmaceuticals, endocrine substances and pesticides. Metals are also being introduced into the environment mainly through mining and copper also via fungicides. With the new and more precise measurement tools available, it is also becoming clearer exactly how strong the contamination of our freshwater systems is.

Heavy metals affect the metabolic activity of organisms, their behaviour and their distribution in the ecosystems and temperature is a very important factor influencing this as well (Lemus & Chung, 1999). Copper is an essential metal for most organisms (Clarkson et al., 1991) since the ions play an important role in their cellular metabolism (Karan et al., 1998). Beyond certain threshold levels (Prato et al., 2013) copper ions are extremely toxic for aquatic organisms and pose a threat to many aquatic organisms when available in excess (De Martinez Gaspar Martins et al., 2011). The target maximum value of the „Amt für Umwelt und Energie, 2009" in Switzerland for copper is 2.0 µg copper per litre.

Copper(II)sulphate pentahydrate $CuSO_4$ * 5 H_2O is a salt in which the five waters of crystallisation give it its bright blue colouring. It is extremely soluble in water with a solubility of 31.6 g / 100 ml water at 0 °C (Weast, 1976). Copper salts are important ingredients in many fungicides and fertilisers used in agriculture (e.g. de Oliveira-Filho et al., 2004) and are one of the most widespread contaminants (Debelius et al., 2009). Copper ions also act as an algaecide and are used in swimming pools or in lakes, ponds and reservoirs to control phytoplankton and aquatic weeds (Effler et al., 1980). Copper-containing fungicides are also used in organic agriculture in Switzerland (Niggli, 2007). Copper ions can enter freshwater systems for example by runoff caused by strong rainfalls. In vineyards where fungicides containing copper ions are commonly applied (e.g. Ruyters et al. 2013), pollution of nearby springs is likely. Copper runoff from vineyard soils is also fortified by wind and water erosion (Komarek et al., 2010). Copper can also enter groundwater and surface waters for example through industrial sewage (Amt für Umwelt und Energie, 2009).

Amphipods: *Gammarus fossarum*

An established approach when investigating the effects of contamination on a certain ecosystem is to make use of a certain species which occurs abundantly and is fairly sensitive towards changes,

but not too sensitive and in the case of most ecotoxicological tests is fairly easy to handle in a laboratory. Therefore we decided to make use of a freshwater organism which occurs abundantly in Swiss headwaters and springs and fulfils the other required criteria as well.

The family Gammaridae belongs to the order Amphipoda (Crustacea). The number of genera placed in the Gammaridae is the subject of much argument and change (Gledhill et al., 1993). Approximately 900 freshwater species in the Gammaridae exist (Gledhill et al., 1993). This family is distributed from the sea of the Arctic to the Antarctic (Schellenberg, 1942). This family represents most freshwater inhabitants, but does not exist in the tropics or South America (Schellenberg, 1942). In the west-Palaearctic region most epigean freshwater species belong to the genus *Gammarus* (Fiser et al., 2012). *Gammarus fossarum* Koch, 1836 (nomenclature according to own nomenclatorial investigations) is a freshwater gammarid species and the most commonly occurring one in Switzerland (Westram et al., 2011). In Switzerland the non-invasive *G. pulex* (Linnaeus, 1758) (nomenclature according to Eggers & Martens, 2004) and *G. roeselii* Gervais, 1835 (nomenclature according to own nomenclatorial investigations) also occur (Rey et al., 2005), albeit less frequently. Headwaters are frequent in Switzerland and *G. fossarum* inhabits these.

To this day at least three cryptic species, types A, B and C, of *G. fossarum* have been identified (Müller, 2000; Westram et al., 2011). These species split during or before the ice ages of the Pleistocene (Webb & Bartlein, 1992). The results of a newly published study reveal that up to 23 overlooked species may be found within *G. fossarum* (Weiss et al., 2014). The biodiversity and biogeography even of one of the most commonly known European amphipod species is still very poorly known (Weiss et al., 2014). However, it has been suggested that type A occurs mainly in eastern European river systems, whereas type B and C are apparently found in the western European regions (Westram et al., 2011). These lineages within *G. fossarum* explain why certain populations do not react entirely in the same manner when exposed to different stressors such as pollutants or unstable environmental conditions. It has been demonstrated that lineage A had significantly higher sensitivity towards teboconazole and thiacloprid than lineage B (Feckler et al., 2012). The type of the gammarids used in the experiments conducted in this PhD project was identified at the Ruhr University of Bochum, Germany. Type B occurs in the spring we took the

gammarids from. The Röserenbach has type A. It can be assumed that both type A and B occur in the spring and the Röserenbach. Another spring, Q3, showed both types.

G. fossarum is an important and efficient shredder (e.g. Schmidt, 2003) and functions as a key species (e.g. Dangles et al., 2004) in the ecosystem, breaking down leaf litter and so linking the terrestrial and aquatic ecosystems (Hieber & Gessner, 2002). *G. fossarum* does not solely break down leaf litter but also feeds on fine particulate organic matter (FPOM) (Moog, 1995). However *G. fossarum* plays a fundamental role in organic matter breakdown in springs and headwaters and in the distribution of coarse particulate organic matter (CPOM) and FPOM (Wagner, 1990; Simcic & Brancelj, 2006). *G. fossarum* is considered a sensitive gammarid species towards contamination of water, low oxygen and low pH (Rinderhagen et al, 2000). It has a relatively wide distribution, mainly in the central and eastern mountainous areas of Europe (Janetzky, 1994; Pöckl et al., 2003). It is not as widely distributed as *G. pulex* (Meijering, 1991; Rinderhagen et al., 2000), but when present, is usually abundantly so. Furthermore, it is a relatively robust representative of the spring fauna and is fairly easy to keep in the laboratory.

Springs: the lower Röseren spring in the Röserental

Springs are relatively temperature-stable (Odum, 1971; van der Kamp, 1995), locally very restricted ecotones between the groundwater and the surface water (Webb et al., 1998). They are physically fragmented ecotones in a terrestrial landscape and as such are particularly vulnerable (Woodward et al., 2010) and can be regarded as early warning systems for environmental changes (Woodward et al., 2009). They can be affected severely by pollutants because of their small size, their isolation and because of the direct connection to the groundwater. Springs can also be influenced strongly by disturbances such as drought or heavy rainfall (von Fumetti & Nagel, 2012). Pollution of springs can occur through entry of contaminated organic matter, run-off of contaminants directly into the water or via the groundwater or via soil erosion. Springs are important habitats for numerous specialised and rare species (Lindegaard et al., 1998) which are necessary for the entire ecotone to function sustainably. Spring species are adapted to the relatively

stable environmental conditions in springs (e.g. Danks & Williams, 1991; Ferrington, 1995), many of them being cold-stenothermal (e.g. Fischer et al., 1998).

Thermal stability seems to be one of the main characteristics of springs fed by deep groundwater (Fischer et al., 1998). This is also thought to be the reason for the presence of cold-stenothermal species (Erman & Erman, 1995). However, there is also evidence for certain variability in the temperature regime of springs (Fischer et al., 1998; von Fumetti et al., 2007) and since Global Change is causing more extreme weather events, the variability of the water temperature will become larger.

The Röserental, near Liestal in the Canton Basel-Landschaft in Switzerland, with an average altitude of 400 m above sea level, has 22 springs (von Fumetti, 2014). These all flow into the small river Röserenbach (von Fumetti et al., 2007). This valley is part of the Swiss Tabular Jura Mountains with the Oxfordian as the main aquifer (von Fumetti, 2008). Although the underground is slightly karstified, most of the springs do not show the characteristics of typical karst springs because of the unkarstified upper geological layers (von Fumetti, 2008). A comprehensive study on the springs in the Röseren valley was conducted by Geijskes (1935). Information on the hydrogeology of these springs can be found in Butscher & Huggenberger (2007). A cultivated forest used for logging, partly consisting of non-resident Thuja, covers a vast part of this valley. Owing to the land use, the nitrate values of some of the springs is elevated, otherwise the water quality of the springs is good.

The spring used in this thesis is called lower Röseren spring (Q4, see von Fumetti & Nagel, 2012), is a natural rheocrene which flows out of a pipe, flows naturally for about 10 meters and then flows back into a pipe below a gravel path. It then flows over a few meters of large blocks of stone into the small river Röserenbach. This spring has a fairly strong and steady discharge and does not dry out in summers. Its fauna is typical of a rheocrene spring in the Swiss Jura (for more information see von Fumetti, 2008 & 2014), with *G. fossarum* occurring abundantly. The fauna of this spring has been monitored over the last 10 years (von Fumetti, 2014) and to date our experiments have not had an impact on the fauna.

Objectives of the thesis

The aim of this thesis is to show the impacts which elevated water temperatures and exposure to copper ions can have on the spring fauna by using *G. fossarum*, a relevant representative of the spring fauna. *G. fossarum* is a spring and head water inhabitant which is not cold stenothermal or crenobiont, but is nevertheless known for its sensitivity and is used as a model organism in many laboratory tests. The experiments which were conducted demonstrate possible scenarios of what could take place in springs under the influence of Global Change and pollution caused by pesticides, pharmaceuticals and other xenobiotics which are brought into the environment.

In order to understand how elevated temperatures could affect the spring fauna, *G. fossarum* specimens were tested in laboratory experiments in a first step. These experiments were conducted in flow channels, and the effects elevated temperatures have on the feeding and ETS activity of *G. fossarum* were examined **(chapter two)**. The aim was to find out how tolerant this species is to temperature changes, considering the ongoing temperature elevations caused by Global Change.

In **chapter three** further experiments with *G. fossarum* were conducted in the laboratory, testing the effects elevated water temperatures combined with exposure to copper ions had on the level of the organism and on the cellular level of the species. The results are discussed with regard to the more specialised and sensitive spring fauna and the implications for spring ecology.

The importance of field experiments is undisputed and yet experiments like this are not very common, owing to many restricting and arduous conditions. Since field experiments are however the most realistic approach when examining the impact of a stressor on an organism, laboratory as well as field experiments using test chambers were conducted in **chapter four**. The same experimental set-up was used in both locations for optimum comparability. We investigated the impact of copper contaminated leaf discs on the feeding and ETS activity of *G. fossarum*. With these experiments we demonstrate the importance of conducting experiments not only in a laboratory but also in nature, as the results do not necessarily have to be similar. The results of our experiments make this clear. The implications for spring inhabitants in general and the relevance of field experiments for future freshwater research are discussed.

References

Amt für Umwelt und Energie (2009) Qualität der Oberflächengewässer im Kanton Basel-Stadt. Untersuchungsjahre 1993 bis 2008. Gewässerschutz. Departement für Wirtschaft, Soziales und Umwelt Basel-Stadt. Zuletzt besucht: 04.02.2015 http://www.aue.bs.ch/dms/aue/download/umweltanalytik/BERICHT2008_OFG_AUE-BS.pdf.

Butscher C, Huggenberger P (2007) Implications for karst hydrology from 3D geological modelling using the aquifer base gradient approach. Journal of Hydrology 342: 184-198.

Carpenter SR, Fisher SG, Grimm NB, Kitchell JF (1992) Global Change and Freshwater Ecosystems. Annual Review of Ecology and Systematics 23: 119-139.

Clarkson TW, Fishbein L, Geldmacher-von Mallinckrodt M, Piscator M, Schlipköter HW, Stoeppler M, Stumm W, Sunderman Jr. FW (1991) Metals and Their Compounds in the Environment. Occurence, Analysis, and Biological relevance. Edited by Merian E. VCH Verlagsgesellschaft mbH, Weinheim.

Dangles O, Gessner MO, Guérold F, Chauvet E (2004) Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. Journal of Applied Ecology 41: 365-378.

Danks HV, Williams DD (1991) Arthropods of springs, with particular reference to Canada: Synthesis and needs for research. In Williams, D. D. & H. V. Danks (eds), Arthropods of springs, with particular reference to Canada. Memoirs of the Entomological Society of Canada 155: 203–217.

Daufresne M, Roger MC, Capra H, Lamouroux N (2003). Long-term changes within the invertebrate and fish communities of the Upper Rhone River: effects of climatic factors. Global Change Biology 10: 124-140.

Debelius B, Forja JM, DelValls A, Lubian L (2009) Toxicity and bioaccumulation of copper and lead in five marine microalgae. Ecotoxicology and Environmental Safety 72: 1503-1513.

Della-Marta PM, Haylock MR, Luterbacher J, Wanner H (2007) Doubled length of western European summer heatwaves since 1880. Journal of Geophysical Research – Atmospheres 112:D15103.

De Martinez Gaspar Martins M, Barcarolli IF, De Menezes EJ, Giacimin MM, Wood CM, Bianchini A (2011) Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectessapidus* acclimated to different salinities: *In vivo* and *in vitro* studies. Aquatic toxicology 101: 88-99.

De Oliveira-Filho EC, Lopes RM, Paumgartten FJR (2004) Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56: 369-374.

Durance I, Ormerod SJ (2007) Climate change effects on upland stream macroinvertebrates over a 25-year period. Global Change Biology 13: 942-957.

Effler SW, Litten S, Field SD, Tong-Ngork T, Hale F, Meyer M, Quirk M (1980) Whole lake responses to low level copper sulphate treatment. Water Research 14: 1489-1499.

Eggers TO, Martens A (2004) Ergänzungen und Korrekturen zum „Bestimmungsschlüssel der Süsswasser-Amphipoda (Crustacea) Deutschlands“. Lauterbornia 50: 1-13, D-86424 Dinkelscherben.

Erman NA, Erman DC (1995) Spring permanence, Trichoptera species richness and the role of drought. Journal of the Kansas Entomological Society 68: 50-64.

Feckler A, Thielsch A, Schwenk K, Schulz R, Bundschuh M (2012) Differences in the sensitivity among cryptic lineages of the *Gammarus fossarum* complex. Science of the Total Environment 439: 158-164.

Ferrington LC (1995) Biodiversity of aquatic insects and other invertebrates in springs: Introduction. Journal of the Kansas Entomological Society 68: 1-3.

Fischer J, Fischer F, Schnabel S, Bohle HW (1998) Spring fauna of the Hessian Mittelgebirge: Population structure, adaptative strategies, and relations to habitats of the macroinvertebrates, as exemplified by springs in the Rhenisch metamorphic shieldand in the East-Hessian sandstone plate. In Botosaneanu, L. (ed.), Studies in crenobiology. The Biology of Springs and Springbrooks. Backhuys Publishers, Leiden: 182-199.

Fiser, Gerecke, Meisch, Stoch (2012) Nationalpark Gesäuse. Quellen. Schriften des Nationalparks Gesäuse 7. Kapitel iv: Milben (Acari) und Krebstiere (Crustacea), Weng im Gesäuse.

Geijskes DC (1935) Faunistisch-ökologische Untersuchungen am Röserenbach bei Liestal im Basler Tafeljura. Beiträge zur Ökologie der Mittelgebirgsbäche. Tijdschrift voor Entomologie 78:249–382.

Gledhill T, Sutcliffe DW, Williams WD (1993) British freshwater Crustacea Malacostraca: a key with ecological notes. Freshwater biological association, scientific publication 52, Ambleside. ISBN 0 900386 53 3.

Haidekker A, Hering D (2008) Relationship between benthic insects (Ephemeroptera, Plecoptera, Coleoptera, Trichoptera) and temperature in small and medium-sized streams in Germany: A multivariate study. Aquatic Ecology 42: 463-481.

Hari RE, Livingstone DM, Siber R, Burkhardt-Holm P, Güttinger H (2006) Consequences of climatic change for water temperature and brown trout populations in Alpine rivers and streams. Global Change Biology 12: 10-26, doi: 10.1111 / j.1365-2486.2005.01051.x

Heino J, Virkkala R, Toivonen H (2009) Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. Biological Reviews 84: 39-54.

Hering D, Haidekker A, Schmidt-Kloiber A, Barker T, Buisson L, Graf W, Grenouillet G, Lorenz A, Sandin L, Stendera S (2010) Monitoring the responses of freshwater ecosystems to climate change. In: Kernan, M., Battarbee, R. W. & Moss, B. (eds), Climate change impacts on freshwater ecosystems. Wiley & Sons, Chichester: 84-118.

Hieber M, Gessner MO (2002) Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology 83:4: 1026-1038.

IPCC 2007. Climate Change (2007). Synthesis Report. Contribution of working Groups 1, 2 and 3 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. (Eds. Pachauri RK, Reisinger A). IPCC, Geneva, Switzerland.

IPCC 2014. Climate Change (2014) Synthesis Report. Longer Report. IPCC Fifth Assessment Synthesis Report. 116 pages.

Janetzky W (1994) Distribution of the genus *Gammarus* (Amphipoda: Gammaridae) in the River Hunte and its tributaries (Lower Saxony, northern Germany). Hydrobiologia 294: 23-34.

Jentsch A, Kreyling J, Beierkuhnlein C (2007) A New generation of Climate-Change Experiments: Events, Not Trends. Frontiers in Ecology and the Environment 5:7: 365-374.

Karan V, Vitorovic S, Tutundzic V, Poleksic V (1998) Functional Enzymes Activity and Gill Histology of Carp after Copper Sulfate Exposure and Recovery. Ecotoxicology and Environmental Safety 40: 49-55.

Kernan M, Battarbee RW, Moss B (2010) Climate Change Impacts on Freshwater Ecosystems. Wiley & Sons, Chichester.

Komarek M, Cadkova E, Chrastny V, Bordas F, Bollinger JC (2010) Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. Environment International 36: 138-151.

Krajick A (2004) All downhill from here? Science 303: 1601-1602.

Lemus MJ, Chung KS (1999) Effect of Temperature on Copper Toxicity, Accumulation and Purification in Tropical Fish Juveniles *Petenia Kraussii* (Pisces: Cichlidae). Caribbean Journal of Science 35: 64-69.

Lexikon der Geowissenschaften, in sechs Bändern (2000) Spektrum Akademischer Verlag Heidelberg- Berlin. Zweiter Band Edu bis Insti, Vierter Band Nord bis Silb, ISBN 3-8274-0421-5.

Lindegaard C, Brodersen KB, Wiberg-Larsen P, Skriver J (1998) Multivariate analyses of macrofaunal communities in Danish springs and springbrooks. Studies in Crenobiology. The Biology of Springs and Springbrooks (Ed. L.Botosaneanu), 201-219. Backhuys Publishers, Leiden, the Netherlands.

Meijering M (1991) Lack of oxygen and low pH as limiting factors for Gammarus in Hessian brooks and rivers. Hydrobiologia 223: 159-169.

Moog O (1995) Fauna Aquatica Austriaca. Wasserwirtschaftskataster, Bundesministerium für Land- und Forstwirtschaft, Wien.

Müller J (2000) Mitochondrial DNA, variation and the evolutionary history of cryptic *Gammarus fossarum* types. Molecular Phylogenetics and Evolution 15: 260-268.

Niggli U (2007) Mythos “bio”. Kommentare zum gleichnamigen Artikel von Michael Miersch in der Wochenzeitung „Die Weltwoche“ vom 20. September 2007.

OcCC (2008). Das Klima ändert – was nun? Der neue UN-Klimabericht (IPCC 2007) und die wichtigsten Ergebnisse aus Sicht der Schweiz. OcCC – Organe consultatif sur les changements climatiques, Bern.

Odum HT (1971) Fundamentals of Ecology. Saunders, Philadelphia, Pennsylvania.

Pöckl M, Webb BW, Sutcliffe DW (2003) Life history and reproductive capacity of *Gammarus fossarum* and *G. roeseli* (Crustacea: Amphipoda) under naturally fluctuating water temperatures: a simulation study. Freshwater Biology 48: 53-66.

Prato E, Parlapiano I, Biandolino F (2013) Sublethal effects of copper on some biological traits of the amphipod *Gammarus aequicauda* reared under laboratory conditions. Chemosphere, 93:6: 1015-1022.

Rey P, Ortlepp J, Küry D (2005) Wirbellose Neozoen im Hochrhein. Ausbreitung und ökologische Bedeutung. Bundesamt für Umwelt, Wald und Lenadschaft BUWAL. Schriftenreihe Umwelt Nr. 380. Gewässerschutz. Bern

Rinderhagen M, Ritterhof J, Zauke G (2000) Crustaceans as Bioindicators. From: Biomonitoring of Polluted Water - Reviews on Actual Topics (A. Gerhardt, ed.), Trans Tech Publications - Scitech Publications, Environmental Research Forum 9:161-194.

Ruyters S, Salaets P, Oorts K, Smolders E (2013) Copper toxicity in soils under established vineyards in Europe: A survey. Science of the Total Environment 443: 470-477.

Schellenberg A (1942) Die Tierwelt Deutschlands und der angrenzenden Meeresteile nach ihren Merkmalen und nach ihrer Lebensweise. Begründet von Professor Dr. Friedrich Dahl. Weitergeführt von Maria Dahl und Professor Dr. Hans Bischoff. 40. Teil: Krebstiere oder Crustacea, IV: Flohkrebse oder Amphipoda. Jena, Verlag von Gustav Fischer.

Schmidli J, Frei C (2005) Trends of heavy precipitation and wet and dry spells in Switzerland during the 20th century. International Journal for Climatology 25: 753-771.

Schmidli J, Schmutz C, Frei C, Wanner H, Schär C (2002) Mesoscale precipitation variability in the region of the European Alps during the 20th century. International Journal of Climatology 22: 1049-1074.

Schmidt J (2003) Wirkung von Umweltchemikalien auf *Gammarus fossarum*. Populationsexperimente und individuenbasiertes Reproduktionsmodell. Dissertation. Technische Universität Dresden, Dresden.

Simcic T, Brancelj A (2006) Effects of pH on electron transport system (ETS) activity and oxygen consumption in *Gammarus fossarum*, *Asellus aquaticus* and *Niphargus sphagnicolus*. Freshwater Biology 51: 686-694.

Van der Kamp RO (1995) The hydrogeology of springs in relation to the biodiversity of spring fauna: a review. in L. C. Ferrington (editor). Biodiversity of aquatic insects and other invertebrates in springs. Journal of the Kansas Entomological Society 68. 4–17

Von Fumetti S (2008) Distribution, discharge and disturbance: new insights into faunal spring ecology. Dissertation. Universität Basel, Basel.

Von Fumetti S (2014) Naturnahe Quellen und ihre Lebensgemeinschaften – Zehn Jahre Forschung im Röserental bei Liestal. Regio Basiliensis 55:3:101-114.

Von Fumetti S, Nagel P, Baltes B (2007) Where a springhead becomes a springbrook - a regional zonation of springs. Fundamental and Applied Limnology 169:1: 37-48.

Von Fumetti S, Nagel P (2012) Discharge variability and its effect on faunistic assemblages in springs. Freshwater Science 31: 647-656.

Wagner R (1990) Influence of Temperature, Photoperiod and Nutrition on Growth and Consumption of *Chaetopteryx Villosa* (Trichoptera). Holarctic Ecology 13: 247-254.

Weast RC (Editor) (1976) Handbook of Chemistry and Physics, 57th Edition, CRC Press, page B-111.

Webb T, Bartlein PJ (1992) Global changes during the last 3 million years: climatic controls and biotic responses. Annual Review of Ecological Systems 23: 141- 173.

Webb DW, Wetzel MJ, Reed PC, Philippe LR, Young TC (1998) The macroninvertebrate biodiversity, water quality, and hydrogeology of ten karst springs in the Salem Plateau of Illinois. In: Botosaneanu, L. (Ed.), Studies in crenobiology: the biology of springs and springbrooks. Backhuys Publishers, Leiden, pp. 39–48.

Weiss M, Macher JN, Seefeldt MA, Leese F (2014) Molecular evidence for further overlooked species within the *Gammarus fossarum* complex (Crustacea: Amphipoda). Hydrobiologia 721: 165-184.

Westram AM, Jokela J, Baumgartner C, Keller I (2011) Spatial Distribution of Cryptic Species Diversity in European Freshwater Amphipods (*Gammarus fossarum*) as Revealed by Pyrosequencing. PLoS ONE 6:8: e23879.

Woodward G, Friberg N, Hildrew AG (2009) The need for scientific rigour in biomonitoring and conservation of fresh waters. Freshwater ecosystems: biodiversity, management and conservation. Hauppage, NY: Nova.

Woodward G, Perkins DM, Brown LE (2010) Climate change and freshwater ecosystems; impacts across multiple levels of organization. Philosophical transactions of the royal society B 365: 2093-2106.

Zappa M, Kan C (2007) Extreme heat and runoff extremes in the Swiss Alps. Natural Hazards and Earth System Sciences 7: 375-389.

CHAPTER TWO

Temperature Effects on the Feeding and Electron Transport System (ETS) Activity of *Gammarus fossarum*

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Abstract
The effects of an increase in water temperature as a direct consequence of Global Change on organisms living in springs and spring brooks have rarely been studied in laboratory experiments. In this study experiments were conducted to test the response of *Gammarus fossarum* Koch, 1836, as an abundant representative of the European spring fauna, to changing water temperatures. The aim was to find out experimentally how *G. fossarum* reacts to varying and increasing water temperatures. The experiments were conducted in flow channels with spring water. In each flow channel *G. fossarum* were placed in boxes with a flow-through system for four weeks. Two analytical methods were applied: The feeding activity of the amphipods was quantified in order to determine the reaction of *G. fossarum* on the level of the organism and the respiratory Electron Transport System (ETS) assay was conducted in order to determine changes in the test organisms on the cellular level. The results show that the feeding activity of *G. fossarum* increased with increasing water temperature, up to an optimum, indicating an increase in their metabolic activity. The ETS activity does not show significant differences at the different temperatures tested. A possible explanation for this is the ability of the organisms to adapt quickly to the changed environmental circumstances.

Keywords

Thermal stress; amphipod; metabolism; respiratory chain; Global Change

Introduction

The earth's environment is under constant change, and the fauna of all ecosystems is hence subjected to these changes. Global Change is causing heat waves and heavy precipitation events in temperate regions (IPCC, 2007) more frequently and such changes in temperature and precipitation are already noticeable (Della-Marta et al., 2007; Hegerl et al., 2011). It has been shown that freshwaters and their biodiversity are especially vulnerable towards Climate Change (e.g. Heino et al., 2009, Woodward et al., 2010). Already in the early 1990ies it was expected that a temperature increase would have a great impact on the structure and functioning of freshwater ecosystems (Carpenter et al., 1992). The water temperature of Swiss rivers has already increased significantly (Vittoz et al., 2013). Springs are relatively temperature-stable ecotones with limited seasonal fluctuations (van der Kamp 1995; Cantonati et al., 2006) and this thermal stability seems to be one of the main characteristics of springs fed by deep groundwater (Fischer et al., 1998). However, there is also evidence for certain variability in the temperature regime of springs (Fischer et al., 1998; von Fumetti et al., 2007) and since Global Change is causing more extreme weather events, the variability of the water temperature will become larger.

Gammarus fossarum Koch, 1836 (Crustacea; Amphipoda) is a relatively robust representative of the macrozoobenthos of springs. Since it inhabits springs and spring brooks abundantly, mainly in the central and eastern mountainous areas of Europe (Janetzky, 1994; Pöckl et al., 2003), it can be considered a suitable organism for assessing possible impacts of Global Change on species inhabiting springs. Furthermore *G. fossarum* plays a fundamental role in organic matter breakdown in springs and spring brooks and hence in the distribution of coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM) (Wagner, 1990; Simcic & Brancelj, 2006). It was chosen as the test organism because of its abundant occurrence, its importance as an efficient shredder (Schmidt, 2003) and its function as a key species in the ecosystem, breaking down leaf litter and so linking the terrestrial and aquatic ecosystems (Hieber & Gessner, 2002). A set of experiments, in which the feeding and respiratory ETS activities of *G. fossarum* at different water temperatures were examined, was conducted.

The feeding activity of organisms has been observed in numerous experiments with gammarids, often on *Gammarus pulex* (Linnaeus, 1758), (e.g. Pascoe et al., 1995; Graca et al., 2001; Felten at al., 2008) but also on *G. fossarum* (e.g. Dedourge-Geffard et al., 2009; Bundschuh et al., 2011), and is suitable to assess for example the effect of low concentrations of pollutants which are found in nature (Pestana et al., 2007). It is influenced by the choice of food i.e. tree species they feed on. Most feeding tests described in literature are conducted with *Alnus glutinosa* leaves (e.g. Maltby et al., 2002; Cold & Forbes, 2004; Bundschuh et al., 2009), these being the preferred tree species of *G. fossarum* and other detritivores (Bloor, 2009).The feeding activity is furthermore a simple way of assessing one aspect of the metabolic activity of the test organisms on the level of the organism.

The respiratory Electron Transport System (ETS) is an enzyme system found in the inner mitochondrial membranes of eucaryotes and controls the oxygen consumption (G.-Toth, 1999). The results obtained from this assay reflect the maximum oxygen consumption when all enzymes are functioning optimally (Kenner et al., 1975). The ETS assay was developed by Packard (1971) and improved by G.-Toth (1999). Originally the biochemical method for measuring respiration via the electron transport system was used to determine oxygen respiration of marine plankton (Packard, 1971). Impairment of the organisms by elevated water temperature may have effects on the functioning of these enzymes and subsequently alter the ETS activity. The ETS assay is a useful tool to assess changes on the cellular level.

The aim of this study was to find out experimentally how *G. fossarum* reacts to varying and increasing water temperatures, which could occur in summer in Swiss springs. The hypotheses of this study were (i) that the feeding activity would increase at higher temperatures and (ii) that the ETS activity would increase with increasing temperature and then decrease if the temperature reached the thermal tolerance limit of *G. fossarum*.

Materials and Methods

Chemicals

Copper sulphate penta-hydrate ($CuSO_4*5H_2O$, Merck, Lot no. A921690 717), magnesium sulphate hepta-hydrate ($MgSO_4*7H_2O$, Merck, Lot no. A966886 729), formaldehyde solution min. 37 % GR (HCHO, Merck, Lot no. K23876703 714), ortho-phosphoric acid 85 % (H_3PO_4, Merck, Lot no. K43024773 150) and potassium dihydrogen phosphate (H_2KPO_4, Merck, Lot no. K23916773 715) were purchased from Merck (Germany). Polyvinyl pyrrolidone (PVP) ($(C_6H_9NO)_x$, Sigma Aldrich, Lot no. BCBG5331V), Triton-X-100 (Triton, Sigma Aldrich, Lot no. BCBC9283V), β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH, Sigma Aldrich, Lot no. 071M7021V), β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADPH, Sigma Aldrich, Lot no. SLBH3107V), 2-p-iodo-phenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT, Sigma Aldrich, Lot no. BCBG6164V) and sodium phosphate dibasic dodeca-hydrate ($HNa_2O_4P*12H_2O$, Sigma Aldrich, Lot no. SZBB201AV), were obtained from Sigma Aldrich (Germany).

Choice of test organisms

Gammarus fossarum specimens were collected from one natural spring, a rheocrene, in the Röserental near Liestal, in Switzerland (for further information see von Fumetti & Nagel (2012)), and transported in spring water with leaves and stones to the laboratory. Individuals of both sexes were collected. Their size ranged from about 8 mm to 15 mm body length, the majority of the gammarids being about 10 mm long. The wet weight of the gammarids used in these experiments ranged from 8 to 14 mg. The selected test specimens showed no visible form of parasitism and moved actively before being used for the experiments. The gammarids were kept at 10 °C for three and a half days for acclimatization to laboratory conditions before being used in an experiment.

Conditioning of leaves

Beech leaves (*Fagus sylvatica* L.) were collected from the litter layer near the spring from which the gammarids were obtained, after abscission in autumn 2011. As the spring is surrounded by

beech trees its leaves form the most important food source for many spring inhabitants including *G. fossarum*. The collected leaves were dried in an oven at 40 °C. They were stored as described by Bloor (2010). Leaf discs (Ø 1 cm) were cut out of the collected leaves with the help of a cork borer. Twenty-eight leaf discs (more info see below) were always weighed together and then placed together in numbered stainless steel herb infusers (Ø 9 cm). This was important for the quantification of the feeding activity, described later on. There are many different ways of conditioning leaves (e.g. Malbouisson et al., 1995; Coulaud et al., 2011) and we decided not to use synthetic water mixtures. The herb infusers containing the leaf discs were submerged in aerated spring water with FPOM from the spring for conditioning for 4 weeks at 17 °C. The temperature was given by the room temperature and was chosen in order for conditioning to be more efficient.

Test design

The experiments were conducted in a laboratory in 4 stainless steel flow channels, with 2 control and 2 experimental channels. Each system consisted of the actual flow channel (1 m x 0.4 m x 0.2 m), a tube through which water flowed into a rain barrel (60 litres) functioning as a water reservoir, an aquarium pump (EHEIM, compact 1000, Deizisau, Germany) to pump the water into the channel and a cooling unit (Aqua Medic Titan 500, Blessendorf, Germany) which helped regulate the water temperature, with an accuracy of ± 0.5 °C. Six plastic boxes (78 mm x 108 mm x 67 mm) from which two sides were removed and replaced by mesh (mesh width 1 mm) in order for water to flow through them but to keep in the test organisms, were placed in every channel. Seven test organisms were placed into each box. Each flow channel held 3 boxes for feeding tests and 3 boxes for the ETS analysis. Each box also held 28 leaf discs so that every gammarid had 1 leaf disc per week to feed on and sufficient foliage for shelter. Natural spring water was used from the spring from which the organisms were collected so that the presence of a natural microflora was given (Jonsson & Malmquist, 2000). The photoperiod was adapted to the season and the time changed once a fortnight to the current sunrise and sunset times. All flow channels were illuminated by two different aquaria-lights (Juwel Aquarium warm-lite and Juwel Aquarium day-lite). The set-up of the experiments in the laboratory was deliberately as near-natural as possible.

The experiments lasted 26 days, during which time the water temperature was varied. The control groups were always kept at 10 °C and the experimental groups were varied for example as follows: first week 10 °C, second and third week 14 °C and last week 14 °C. In some regimes the water temperature was decreased back to 10 °C in the fourth week to see if the organisms would recover from the temperature elevation (Tab. 1). Many laboratory studies with gammarids use water temperatures of 12 to 16 °C (e.g. Cold & Forbes, 2004; Bundschuh et al., 2009; Schaller et al., 2010; Coulaud et al., 2011). This seems rather high for *G. fossarum* collected from springs, which are usually found in springs and spring brooks in Switzerland with temperatures around 10 °C all year round, with absolute peaks of at most up to 14 °C (von Fumetti & Nagel, 2012). Although it is unlikely that the water of the Swiss spring used in this study will ever reach temperatures of 18 °C, this temperature was chosen in order to ascertain the tolerance of the tested individuals of this species. The temperature changes were achieved within 24 hours.

For the duration of the experiments oxygen saturation (%) and concentration (mg / L), pH and electrical conductivity (µS / cm) of the spring water in the flow channels were measured twice a week, using portable meters (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). The phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) concentrations (all in mg / L), of the spring water in the flow channels were measured photometrically twice a week (Spectroquant NOVA 60, Merck, Darmstadt, Germany) to ensure that the water quality was stable.

Feeding activity

The organisms were monitored regularly for deaths and dead gammarids were removed and preserved in 100 % ethanol. At the end of the experiments all gammarids were preserved in 100 % ethanol and subsequently dried at 40 °C and then weighed. The leaf discs they had fed on were also dried at 40 °C and weighed. Additional replicates were set up to control for leaf mass loss driven by abiotic factors and microbial decomposition. The results of these were used for the quantification of the feeding activity (= control factor). *Gammarus fossarum* is a shredder, and hence not all processed i.e. shredded leaf material is actually eaten, therefore we refrain from using the term feeding rate and instead use the general term feeding activity. A feeding activity was

determined for every box, for the 7 organisms together, as described by Maltby et al. (2002) after the 26 days experiment duration:

$$FA = ((Li \times \text{Control factor}) - Lf) / (\text{weight} \times \text{time})$$

where FA is the feeding activity, Li is the initial leaf weight (mg), Control factor is the loss of weight of leaves during four weeks when no feeding takes place, Lf is the final leaf weight (mg), weight is the dry weight of the gammarids (mg) and time is the duration of the experiment (days).

ETS activity

The ETS analysis was conducted as described by Simcic & Brancelj (1997). ETS assays were carried out at the end of weeks one, three and four in order to detect the influence of the changing water temperatures on the organisms at the cellular level. For each experiment 72 individuals were analysed, 24 individuals per week, i.e. 12 individuals from the control and 12 from the experimental group. Six live gammarids were removed from the flow channels one by one using tweezers, placed on a tissue and gently patted dry. They were then weighed singly on a microbalance (XP6, METTLER TOLEDO, Greifensee, Switzerland), placed on a numbered aluminium sheet with some distilled water. The gammarids were then homogenised singly in the homogenisation tube without the distilled water, using 4 ml of ice-cold homogenising buffer solution [0.1 M sodium phosphate buffer pH 8.4, 75 µM $MgSO_4$, 0.15 % (w/v) polyvinyl pyrrolidone, 0.2 % (v/v) Triton-X-100]. The homogenate was poured into a centrifuge tube and sonicated with an ultrasonic homogeniser (Bandelin Sonopuls HD2070, Berlin, Germany) for 20 seconds and stored in an ice solution. The homogenate was then centrifuged (Sigma 2-16 PK, Osterode am Harz, Germany) at 0 °C for 4 minutes at 10000 r.p.m., according to Simcic & Brancelj (2004). The supernatant (in triplicate) was incubated with 1.5 mL substrate solution [0.1 M sodium phosphate buffer pH 8.4, 1.7 mM NADH, 0.25 mM NADPH, 0.2 % (v/v) Triton-X-100] and 0.5 mL reagent solution [2.5 mM 2-p-iodo-phenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride] for 40 minutes at 10 °C. Stopping solution [formaldehyde (conc.):H_3PO_4 (conc.) = 1:1],

0.5 mL, was added immediately after incubation and the formazan production determined spectrophotometrically with the spectroquant ® Pharo 300 (Merck, Darmstadt, Germany) by measuring the absorbance of the sample at 490 nm against the blank.

The ETS activity was calculated according to Kenner & Ahmed (1975):

$$\text{ETS activity } (\mu L\ O_2 / mg \times h) = ((Abs^{490nm} \times Vr \times Vh \times 60) / (Va \times Gw \times t \times 1.42)$$

where Abs^{490nm} is the absorption of the sample, Vr is the final volume of the reaction mixture (3 mL), Vh is the volume of the original homogenate (4 mL), Va is the volume of the aliquot of the homogenate (0.5 mL), Gw is the gammarid wet weight (mg), t is the incubation time (minutes) and 1.42 is the factor for conversion to volume oxygen.

Data analysis

The analyses of variances (ANOVAs) were calculated using the Statistical Package for Social Sciences (SPSS) version 21 for Windows (SPSS Inc, Chicago, IL, U.S.A.). One-way ANOVAS were conducted to test the influence of the water temperatures on the feeding and ETS activities of the test organisms.

Variance homogeneity was tested with the Levene-Test. The feeding activity data was found to be homogeneous. To correct for multiple single comparisons a Bonferroni-Holm correction was applied. For the ETS data variance was found to be unequal. A reason for this is because very small sample sizes may be particularly sensitive to the homogeneity of variance assumption. A Games-Howell-Test, which is applied in such cases of unequal variances and also takes unequal group sizes into account, was applied to the ETS data. The α-level was set at 0.05.

Results

The results demonstrate higher feeding activities at higher temperatures, being highest in condition 10-18-18-18° C. The lowest measured feeding activity was 0.03 mg / mg × d at 10 °C; the highest one was 3.37 mg / mg × d, measured at 18 °C.

An ANOVA comparing the different temperature regimes revealed a significant effect on the feeding activity, $F_{4, 67} = 12.537$, $p < 0.001$. Further single comparisons were calculated using the Bonferroni-Holm correction. The feeding activities in the control (M = 0.09, SD = 0.04) were significantly lower than in conditions 10-14-14-14 °C (M = 0.14, SD = 0.04) and 10-18-18-18 °C (M = 0.20, SD = 0.09) ($p < 0.005$ and $p < 0.001$, respectively) (Fig. 1a). No significant differences could be made out between the control and conditions 10-14-14-10 °C (M = 0.09, SD = 0.03) and 10-18-18-10 °C (M = 0.07, SD = 0.01) ($p = 1.0$ and $p = 1.0$, respectively) (Fig. 1b).

Although 83.7 % of the organisms died in condition 10-18-18-18 °C (Tab. 1), the surviving gammarids shredded significantly more than those in the control. Significant differences also exist between condition 10-14-14-10 °C (M = 0.09, SD = 0.03) and 10-14-14-14 °C (M = 0.14, SD = 0.04) ($p = 0.040$). Lastly, the difference between condition 10-18-18-10 °C (M = 0.07, SD = 0.01) and 10-18-18-18 °C (M = 0.20, SD = 0.09) was found to be significant ($p < 0.001$).

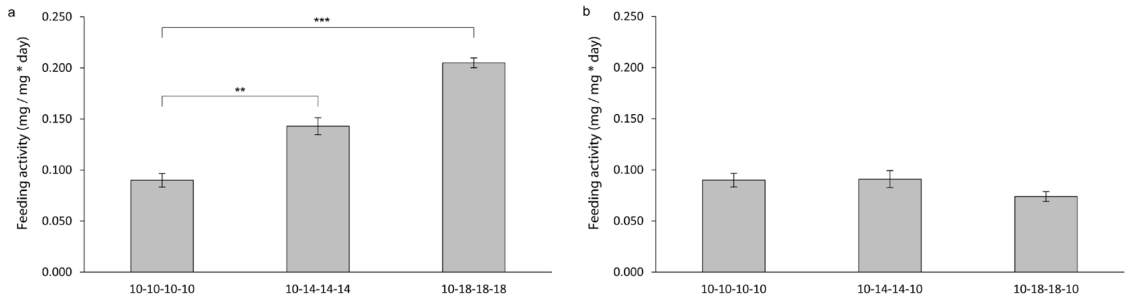

Fig. 1a & 1b Mean feeding activities at the different conditions. The control is always at 10 °C, the experimental temperature regimes range between 10 °C and 18°C. Standard errors are represented in the figure by the error bars attached to each column. Note: *** < 0.001, ** < 0.005

ETS activity

Generally mean ETS values are in a similar range. The lowest measured ETS activity was 0.105 µL O_2 / mg × h, the highest one was 1.142 µL O_2 / mg × h, both measured at 10° C.

An ANOVA comparing the different temperature regimes revealed no significant effect on the ETS activity, $F_{7, 360} = 1.831$, $p = 0.080$. When looking at the conditions which lasted 3 weeks and then at

the conditions which lasted 4 weeks, it can be noted that a bell-shaped curve could be drawn over the three bars (Fig. 2a & 2b).

In condition 10-18-18-18 °C the organisms destined for ETS analysis had died before the end of week 4. The reason for the many deaths (83.7 %) in this condition was probably entirely owing to the high temperature of 18 °C, with which the test organisms were not able to cope for longer than 3 weeks. In the other conditions the average death rate was around 30 % (Tab. 1).

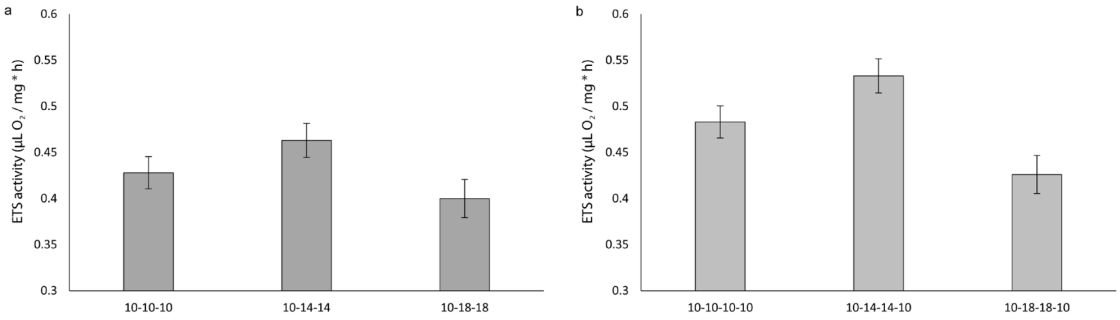

Fig. 2a & 2b Mean ETS activities of the conditions lasting 3 weeks (a) and those lasting 4 weeks, with the temperature regimes that declined after 3 weeks back to 10 °C (b). The control conditions are always at 10 °C, the experimental temperature regimes range between 10 °C and 18 °C. Standard errors are represented in the figure by the error bars attached to each column.

Table 1: Temperature regimes applied in the conducted experiments. Control groups were always kept at 10° C for the entire experiment. The date indicates the start of the experiment. Deaths during the experiments are given in percentage.

Date of experiment beginning (2012) Exp. 1 - 6	Temperature regime = conditions [°C] weeks 1 –2 – 3 – 4	deaths control groups [%]	deaths experimental groups [%]
5th March	10-14-14-10	39.3	17.9
2nd April	10-14-14-14	7.1	8.3
30th April	10-18-18-10	7.1	33.3
28th May	10-18-18-18	45.1	83.7
25th June	10-14-14-14	65.3	55.1
23rd July	10-14-14-10	42.9	45.2

Discussion

Feeding activity of *G. fossarum*

The conducted feeding tests demonstrate that higher temperatures increase the feeding activity of *G. fossarum* and therefore have an impact on *G. fossarum* on the organismal level. Higher temperatures generally cause higher metabolic rates (e.g. Georgiadis, 1977). In addition it has also been observed that the consumption of stream-water conditioned alder leaves by *G. pulex* increased with increasing temperatures (Malbouisson et al., 1994). Feeding tests conducted with *G. pulex* at 2, 5, 10 and 15 °C with *Alnus* sp. and *Fagus* sp. leaves caused a rise in consumption with increasing temperature (Nilsson, 1974). It would appear in this study that the conditions with the temperature regimes involving 14 °C were not stressful for the gammarids but might even be considered more optimal for these organisms. The feeding activity in the conditions 10-14-14-10 °C and 10-18-18-10 °C were not significantly higher than the feeding activity of the control. It can be argued that a decrease in temperature after 2 weeks of an elevated water temperature normalizes the feeding activity so that the overall feeding activity ends up not significantly different from the feeding activity in the control condition. In this case a decrease of water temperature after the initial elevation has a stabilizing effect on the organisms' metabolism. It was assumed that higher temperatures would raise mortality; indeed this was the case at 18 °C. This temperature was too warm for the gammarids, and the surviving ones were stressed by an elevation of their metabolic activity.

The sex of the test organisms can influence the feeding activity (Malbouisson et al., 1994). In all the experiments females and males were used without determining their sexes, since determination would cause unnecessary stress of the test organisms. Also, we aimed at conducting the experiments as near-naturally as possible. However, it was always ensured that specimens of similar size compositions were used in the experiments. For *G. pulex* it has been shown that females consumed less food than males in terms of absolute dry weight (Malbouisson et al., 1994). It can be assumed that females of *G. fossarum* behave likewise and therefore certain observed feeding activities are potentially lower than others owing to the sex of the organisms used. However, it should be noted that natural differences in appetites of individuals can also have an

effect on the feeding activity (Taylor et al., 1993). Certain results may also be affected by the fact that some test organisms moulted during the tests. In those cases it was observed that they often died within the following 24 hours. *Gammarus fossarum* specimens were also used regardless of the time of their last moult in tests conducted by Simcic & Brancelj (2003).

ETS activity of *G. fossarum*

The measured ETS activities in this study are in a similar range to ETS activities measured by other authors (e.g. Simcic et al., 2005; Simcic & Brancelj, 2006; Lukancic et al., 2010). It was assumed that ETS activity, i.e. the enzyme activity, would increase with increasing temperatures as demonstrated by Simcic and Germ (2010) up to a turning point and then decrease.

The ETS activities measured after 3 weeks, comparing the temperature regimes 10-10-10 °C, 10-14-14 °C and 10-18-18 °C, were not significantly different. However it would appear that the ETS activity increased in the condition 10-14-14 °C but decreased in the condition 10-18-18 °C, compared to the mean ETS activity of the control channel kept at 10 °C during the 3 weeks. The same pattern is observed when comparing the ETS activities measured after 4 weeks, comparing the temperature regimes 10-10-10-10 °C, 10-14-14-10 °C and 10-18-18-10 °C. In natural environments *G. fossarum* competes with *G. pulex*. *Gammarus pulex* is more robust towards higher water temperatures up to 27 °C (Sutcliffe et al., 1981; Foucreau et al., 2014) and so *G. fossarum* is forced into colder waters. An intermediate temperature such as 14° C is probably more suitable for *G. fossarum* than 10 °C. However, higher water temperatures than 14 °C, for example 18 °C, were shown to have detrimental effects on *G. fossarum*, which could lead this species and other more sensitive species into survival difficulties.

In order to understand the results obtained in this study, it needs to be pointed out that the ETS activities of the gammarids in this study were measured one week after changing the water temperature. This gave the gammarids a week to adapt to the new temperature: probably long enough for them to recover and stabilize their normal ETS activity. This adaption would explain the similar values of the control to the experimental conditions. In an experiment with daphnids at different temperatures, an adaptation time to experimental temperatures of only 3 hours was given

(Simcic & Brancelj, 1997). It has been shown that a certain time span enables adaptation of the species to the new circumstances (e.g. Bamsted, 1980). Organisms which live in relatively stable temperature environments, such as spring species, are not well adapted to temperature fluctuations. In such cases ETS activity is more sensitive to temperature variations (Simcic & Brancelj, 1997). Therefore it can be assumed that *G. fossarum* will react in a similar way when little time to adapt is given. It can also be assumed that the observed pattern, an increase at the intermediate temperature regime with 14 °C but a decrease at the extreme temperature regime with 18 °C, would become significant if adaptation time was considerably shortened. This will be a subject for future investigation. The findings also give an insight into the high adaptability of *G. fossarum* towards intermediate temperature elevations. The results do not suggest that a gradual temperature increase on its own, caused by Global Change, will be as problematic for *G. fossarum* on the cellular level as initially thought.

Implications on the population and community level

Water temperature has a dominating influence on the life history, reproduction and growth of *G. fossarum* (Pöckl et al., 2003). In this study it was shown that higher water temperatures caused higher feeding activities. This has consequences for *G. fossarum* on the **population level**, as a higher feeding activity induced by higher water temperatures as a direct consequence of Global Change could result in a faster depleted food source. Springs are dependent on allochtonous material input. For example: the spring the gammarids were taken from for this study is situated in a forest with a limited number of trees surrounding it. By the end of summer hardly any leaf litter is left in the springs. If the water temperature rises as predicted, and the feeding activity rises as observed in this study, then food might become scarce by the end of summer and so become a limiting factor for the organisms living in these ecotones. This food shortage in return could result in the reduction or complete stop of the individual's scope for growth. This in turn might decrease the amount of energy available for reproduction and decreases the individual's probability of survival (Naylor et al., 1989; Maltby, 1992).

On the **community level**, *G. fossarum* acts as a shredder, breaking down CPOM to FPOM (Wagner, 1990; Simcic & Brancelj, 2006). Therefore it plays a very important role in the lotic food web of springs, enabling other species to survive on the FPOM provided. Increased water temperatures could therefore cause larger FPOM production. This may be positive for species dependent on that food source, given that they survive the elevated water temperatures.

According to the results 14 °C seems to pose no serious problems for *G. fossarum*, but other species living in springs might be affected more strongly by such an increase in water temperature, because they are adapted to a relatively stable environment. In consequence, elevated water temperature could change whole species assemblages and alter the susceptibility of macroinvertebrates to environmental stressors such as pesticides, pharmaceuticals or illnesses. A water temperature of 14 °C has indeed been measured in the spring used in this study in summer during especially warm periods.

Generally, elevated water temperatures may cause physiological stress, rising metabolic activity and in consequence reduced fitness (Hering et al., 2010). The body temperature of most invertebrates fluctuates with the temperature of their immediate environment; subsequently the ETS activity of these species must function under a wide range of temperatures (Simcic et al., 2014). This requirement is however not met by cold water adapted species. Supporting this, it has been demonstrated that cold-stenothermal chironomids had a lower ETS activity at high water temperatures (Simcic, 2005).

In response to Global Change species are already expanding their ranges to higher latitudes and altitudes (Krajick, 2004; Heino et al., 2009). Especially cold-stenothermal species will experience strong range shifts or range contractions, as space with suitable thermal conditions decreases (Woodward et al., 2010). Extreme weather events in Europe are becoming more frequent owing to Global Change and so temperature fluctuations such as those used in these experiments will become increasingly important.

Conclusion

By studying the feeding and the ETS activity of *G. fossarum* exposed to elevated water temperatures, the aim was to benefit from the simplicity of the one method on the level of the organism and complexity of the other method on the cellular level. Although the approach of these two methods is different, the results obtained by them should be complementary in assessing the responses of the test organisms. The feeding activity of *G. fossarum* at elevated water temperatures increased significantly. The measured ETS activities were not significantly different at increased temperatures. Reasons why the ETS activities of the gammarids did not increase significantly are discussed. With the results of these experiments it can be concluded that *G. fossarum* is suitable to help evaluate changes occurring in spring ecotones. It was shown that *G. fossarum* is able to adapt to short-term intermediate temperature elevations quickly and is hence not necessarily threatened by moderately rising water temperatures. Moreover, Global Change is interacting with other stressors, for example pesticides, (Durance & Omerod, 2007) and will result in complex cause-effect chains as many other environmental parameters are linked to temperature (Hering et al., 2010). Future research will concentrate on tests with *G. fossarum* under the influence of an additional stressor, with less time being allowed for adaption to the changed environmental circumstances.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Bamsted U (1980) ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. J of Experimental Mar Biol and Ecology 42:267-283.

Bloor M (2009) Aquatic Pollution. Case Study of Landfill Leachate Toxicity and Remediation. VDM Verlag DR. Müller Aktiengesellschaft & Co. KG, Saarbrücken.

Bloor MC (2010) Animal standardisation for mixed species ecotoxicological studies: Establishing a laboratory breeding programme for *Gammarus pulex* and *Asellus aquaticus*. Zoologica Baetica 21:179-190.

Bundschuh M, Hahn T, Gessner MO, Schulz R (2009) Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. Environ Toxicol and Chem 28:197-203.

Bundschuh M, Zubrod JP, Kosol S, Maltby L, Stang C, Duester L, Schultz R (2011) Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquatic Toxicol 104:32-37

Cantonati M, Gerecke R, Bertuzzi E (2006) Springs of the Alps - sensitive ecosystems to environmental change: from biodiversity assessments to long-term studies. Hydrobiologia, 562:59-96

Carpenter SR, Fisher SG, Grimm NB, Kitchell JF (1992) Global Change and Freshwater Ecosystems. Annu Rev of Ecol and Systematics 23:119-139

Cold A, Forbes VE (2004) Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. Aquatic Toxicol 67:287-299

Coulaud R, Geffard O, Xuereb B, Lacaze E, Quéau H, Garric J, Charles S, Chaumot A (2011) In situ feeding assay with *Gammarus fosssarum* (Crustacea): Modelling the influence of confounding factors to improve water quality biomonitoring. Water Res 45:6417-6429

Dedourge-Geffard O, Palais F, Biagianti-Risbourg S, Geffard O, Geffard A (2009) Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: An *in situ* experiment. Chemosphere 77:1569-1576

Della-Marta PM, Haylock MR, Luterbacher J, Wanner H (2007) Doubled length of western European summer heatwaves since 1880. J of Geophysical Res – Atmospheres 112:D15103.

Durance I, Ormerod SJ (2007) Climate change effects on upland stream macroinvertebrates over a 25-year period. Glob Change Biol 13:942-957.

Felten V, Charmantier G, Mons R, Geffard A, Rousselle P, Coquery M, Garric J, Geffard O (2008) Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquatic Toxicol 86:413-425

Fischer J, Fischer F, Schnabel S, Bohle HW (1998) Spring fauna of the Hessian Mittelgebirge: Population structure, adaptative strategies, and relations to habitats of the macroinvertebrates, as exemplified by springs in the Rhenisch metamorphic shieldand in the East-Hessian sandstone plate. In Botosaneanu, L. (ed.), Studies in crenobiology. The Biology of Springs and Springbrooks. Backhuys Publishers, Leiden: 182-199.

Foucreau N, Cottin D, Piscart C, Hervant F (2014) Physiological and metabolic responses to rising temperature in *Gammarus pulex* (Crustacea) populations living under continental or Mediterranean climates. Comparative Biochem and Physiol, Part A 168:69-75

Georgiadis G (1977) Die Auswirkung verschiedener Temperatur- und Salinitätskombinationen auf *Gammarus fossarum* Koch, 1835. Crustaceana Supplement, Number 4, Studies in Gammaridae (Proceedings of the 3rd International Colloquium on *Gammarus* and *Niphargus*, Schlitz, 1975), 112-119

Graca MAS, Cressa C, Gessner MO, Feio MJ, Callies KA, Barrios C (2001) Food quality, feeding preferences, survival and growth of shredders from temperate and tropical streams. Freshw Biol 46:947-957

G.-Toth L (1999) Aktivität des Elektronentransportsystems. In: Biologische Gewässeruntersuchung. Methoden der Biologischen Wasseruntersuchung 2 (Eds W. von Tümpling & G. Friedrich), pp. 465–473. Gustav Fischer Verl., Jena, Stuttgart, Lübeck, Ulm.

Hegerl G, Hanlon H, Beierkuhnlein C (2011) Elusive extremes. Nat Geoscience 4:142-143

Heino J, Virkkala R, Toivonen H (2009) Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. Biological Rev 84:39-54

Hieber M, Gessner MO (2002) Contribution of stream detritivores, fungi, and bacteria to leaf breakdown based on biomass estimates. Ecology 83:1026-1038

Hering D, Haidekker A, Schmidt-Kloiber A, Barker T, Buisson L, Graf W, Grenouillet G, Lorenz A, Sandin L, Stendera S (2010) Monitoring the responses of freshwater ecosystems to climate change. In: Kernan, M., Battarbee, R. W. & Moss, B. (eds), Climate change impacts on freshwater ecosystems. Wiley & Sons, Chichester: 84-118.

IPCC 2007. Climate Change, 2007. Synthesis Report. Contribution of working Groups 1, 2 and 3 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. (Eds. Pachauri, R. K. & Reisinger, A.). IPCC, Geneva, Switzerland.

Janetzky W (1994) Distribution of the genus *Gammarus* (Amphipoda: Gammaridae) in the River Hunte and its tributaries (Lower Saxony, northern Germany). Hydrobiologia 294:23-34

Jonsson M, Malmquist B (2000) Ecosystem process rate increases with animal species richness: evidence from leaf-eating, aquatic insects. Oikos 89:519-523

Kenner RA, Ahmed SI (1975) Measurements of electron transport activities in marine phytoplancton. Mar Biol 33:119-127

Krajick A (2004) All downhill from here? Science 303:1601-1602.

Lukancic S, Zibrat U, Mezek T, Jerebic A, Simcic T, Brancelj A (2010) A new method for early assessment of effects of exposing two non-target crustacean species, *Asellus aquaticus* and *Gammarus fossarum*, to pesticides, a laboratory study. Toxicol and Industrial Health 26:217-228

Malbouisson JFC, Young TWK, Bark AW (1994) Disruption of Precopula in *Gammarus Pulex* as a Result of Brief Exposure to Gamma-Hexachlorocyclohexane (Lindane). Chemosphere 28:2011-2020

Malbouisson JFC, Young TWK, Bark AW (1995) Use of feeding rate and repairing of precopulatory *Gammarus pulex* to assess toxicity of Gamma-Hexachlorocyclohexane (Lindane). Chemosphere 30:1573-1583

Maltby L, Clayton SA, Wood RM, McLoughlin N (2002) Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. Environmental Toxicol and Chem 21:361-368

Maltby L (1992) The use of the physiological energetics of *Gammarus pulex* to assess toxicity: a study using artificial streams. Environmental Toxicol and Chem 11:79-85

Naylor C, Maltby L, Calow P (1990) Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. Hydrobiologia 188/189:517-523

Nilsson, LM (1974) Energy Budget of a Laboratory Population of *Gammarus pulex* (Amphipoda). Oikos 25:35-42

Packard TT (1971) The measurement of respiratory electron transport system activity in marine phytoplankton. J of Mar Res 29:235-244

Pascoe D, Kedwards TJ, Blockwell SJ, Taylor EJ (1995) *Gammarus pulex* (L.) Feeding Bioassay – Effects of Parasitism. Bull of Environmental Contam Toxicol 55:629-632

Pestana JLT, Re A, Nogueira AJA, Soares AMVM (2007) Effects of Cadmium and Zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68:1556-1562

Pöckl M, Webb BW, Sutcliffe DW (2003) Life history and reproductive capacity of *Gammarus fossarum* and *G. roeseli* (Crustacea: Amphipoda) under naturally fluctuating water temperatures: a simulation study. Freshw Biol 48:53-66

Schaller J, Mkandawire M, Dudel EG (2010) Heavy metals and arsenic fixation into freshwater organic matter under *Gammarus pulex* L. influence. Environmental Pollut 158:2454-2458

Schmidt J (2003) Wirkung von Umweltchemikalien auf *Gammarus fossarum*. Populationsexperimente und individuenbasiertes Reproduktionsmodell. Dissertation. Technische Universität Dresden, Dresden 1-10

Simcic T, Brancelj A (1997) Electron transport system (ETS) activity and respiration rate in five *Daphnia* species at different temperatures. Hydrobiologia 360:117-125

Simcic T, Brancelj A (2003) Estimation of the proportion of metabolically active mass in the amphipod *Gammarus fossarum*. Freshw Biol 48:1093-1099

Simcic T, Brancelj A (2004) Respiratory electron transport system (ETS) activity as an estimator of the thermal tolerance of two *Daphnia* hybrids. J of Plankton Res 26:525-534

Simcic T (2005). Respiratory electron transport system (ETS) activity and respiration rate in cold-stenothermal and eurythermal chironomid larvae from high-mountain lakes. Archiv für Hydrobiologie 162:399-415.

Simcic T, Lukancic S, Brancelj A (2005) Comparative study of electron transport system activity and oxygen consumption of amphipods from caves and surface habitats. Freshw Biol 50:494-501

Simcic T, Brancelj A (2006) Effects of pH on electron transport system (ETS) activity and oxygen consumption in *Gammarus fossarum*, *Asellus aquaticus* and *Niphargus sphagnicolus*. Freshw Biol 51:686-694

Simcic T, Germ M (2010) Increased Temperature Due to Global Warming Alters the Respiratory Potential in Aquatic Organisms from an Oligotrophic Lake. Research Paper. Internat Rev of Hydrobiol 95:370-382

Simcic T, Pajk F, Jaklic M, Brancelj A, Vrezec A (2014) The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. J of Therm Biol 41:21-30

Sutcliffe DW, Carrick TR, Willoughby LG (1981) Effects of diet, body size, age and temperature on growth rates in the amphipod *Gammarus pulex*. Freshw Biol 11:183-214.

Taylor EJ, Jones DPW, Maund SJ, Pascoe D (1993) A New Method for Measuring the Feeding Activity of *Gammarus Pulex* (L.). Chemosphere 26:1375-1381

Van der Kamp RO (1995) The hydrogeology of springs in relation to the biodiversity of spring fauna: a review. 4–17 in L. C. Ferrington (editor). Biodiversity of aquatic insects and other invertebrates in springs. J of the Kans Entomological Soc 68.

Vittoz P, Cherix D, Gonseth Y, Lubini V, Maggini R, Zbinden N, Zumbach S (2013) Climate change impacts on biodiversity in Switzerland: A review, J of Nat Conservation 21:154-162

Von Fumetti S, Nagel P, Baltes B (2007) Where a springhead becomes a springbrook - a regional zonation of springs. Fundamental and Appl Limnology 169:37-48

Von Fumetti S, Nagel P (2012) Discharge variability and its effect on faunistic assemblages in springs. Freshw Sci 31:647-656

Wagner R (1990) Influence of Temperature, Photoperiod and Nutrition on Growth and Consumption of *Chaetopteryx Villosa* (Trichoptera). Holarctic Ecol 13:247-254

Woodward G, Perkins DM, Brown LE (2010) Climate change and freshwater ecosystems; impacts across multiple levels of organization. Philosophical Transactions of the R Soc B 365:2093-2106

CHAPTER THREE

Effects of Increased Temperatures on *Gammarus fossarum* under the Influence of Copper Sulphate

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Abstract
The specialised fauna of freshwater springs will have to cope with a possible temperature rise owing to Global Change. It is affected additionally by contamination of the water with xenobiotics from human activities in the surrounding landscape. We assessed the combined effects of temperature increase and exposure to toxins in laboratory experiments by using copper sulphate as a model substance and *Gammarus fossarum* Koch, 1835, as the model organism. This amphipod is a common representative of the European spring fauna and copper ions are widespread contaminants, mainly from agricultural practice. The experiments were conducted in boxes placed in flow channels and the water temperatures were varied. The gammarids were fed with conditioned beech leaf discs. The feeding activity of the amphipods was quantified on the level of the organism; and the respiratory Electron Transport System (ETS) assay was conducted in order to determine changes on the cellular level in the test organisms. The results show that the feeding activity increased slightly with higher water temperature. The sub-lethal copper dose had no significant effect other than a trend towards lower feeding activity. The ETS activity was significantly higher at the higher water temperatures, and the copper ions significantly lowered the ETS activity of the organisms. The combination of the two methods was useful when testing for combined effects of environmental changes and pollutants on a species. From the results one can reasonably infer a higher risk of adverse effects with increase in water temperature and exposure to a particular heavy metal.

Keywords

Thermal stress, amphipod, copper sulphate, feeding activity, electron transport system (ETS)

Introduction

Springs are special ecotones between the groundwater and the surface water (Webb et al. 1998) and are important habitats for numerous specialised and rare species (Lindegaard et al. 1998) which are necessary for the entire ecotone to function sustainably. Freshwaters such as springs are spatially constrained and can be regarded as early warning systems (Woodward et al. 2009). They can be strongly influenced by pollutants because of their small size, their isolation and because of the direct connection to the groundwater. Spring species are adapted to the relatively stable environmental conditions in springs (e.g. Danks & Williams 1991; Ferrington 1995), many of them being cold-stenothermal (e.g. Fischer et al. 1998). Global Change is causing an increase in temperature, and since a few years extreme events such as heat waves and floods are becoming more frequent in the temperate regions (IPCC 2007). It has been shown that freshwaters and their biodiversity are especially vulnerable towards Global Change (e.g. Heino et al. 2009; Woodward et al. 2010). The water temperature affects macroinvertebrates directly, especially if they are cold-water-adapted species (Heino et al. 2009). It is therefore important to investigate the effects of elevated water temperatures on macroinvertebrates, using realistic temperature scenarios which could occur in nature. This is becoming more important because extremely warm and dry summers are occurring more frequently (Vittoz et al. 2013), so the organisms need to adapt to fast elevating temperatures. Furthermore water temperature raises the toxicity of substances such as fungicides for non-target organisms (Bat et al. 2000; Holmstrup et al. 2010), making the organisms more susceptible to environmental warming. These interactions are of interest in ecotoxicology, since temperature has a large impact on species, especially in combination with toxicants. It is therefore important to conduct tests combining both stressors.

Copper salts are important ingredients in many fungicides and fertilisers used in agriculture (e.g.de Oliveira-Filho et al. 2004) and are one of the most widespread contaminants (Debelius et al. 2009). They also act as an algaecides and hence are used for control of phytoplankton and aquatic weeds (e.g. Effler et al. 1980). Copper salts have been introduced into water bodies as aquatic molluscicides (e.g. Guida et al. 2008). Although a certain amount of copper ions are essential for most organisms, they pose a threat to many aquatic organisms in general when available in excess

in water (De Martinez Gaspar Martins et al. 2011). They are strong fish poisons as well as one of the most toxic metals for microalgae, being toxic at concentrations as low as 1 µg/L (Debelius et al. 2009). Terrestrially applied pesticides can be flushed into springs and rivers through means of runoff. This will take place more regularly when heavy rainfalls occur more frequently. In vineyards where fungicides containing copper ions are commonly applied (e.g. Ruyters et al. 2013), pollution of nearby springs is likely. Although copper salts in soils are strongly immobilized, there has also been evidence of them migrating through soil profiles in vineyards (Komarek et al. 2010). Groundwater quality is then under risk and hence spring water quality is also affected. It is, however, more likely that springs are contaminated via runoff of freshly applied fungicides, intensified by heavy rainfall.

Amphipods mainly take up ions such as Cu^{2+} via their gills since these are a large adsorptive organ system (Reichmuth et al. 2010). This makes them especially susceptible to water-borne pollutants (Rinderhagen et al. 2000). Previous studies have been conducted on the effects of copper salts for example on *G. pulex* (e.g. Taylor et al. 1998; Güven et al. 1999; Brooks & Mills 2003). In a study testing the effects of copper ions on the feeding rate and digestive enzymes of *G. fossarum* it was found that organisms exposed to a metallic contaminated site had inhibited digestive enzymes and a decreased feeding activity (Dedourge-Geffard et al. 2009).The impact of Cu^{2+} is expected to be stronger at higher temperatures because the higher temperatures can cause increased breathing as a consequence of the elevated metabolic activity and hence increased absorption of ions (Lemus & Chung 1999). Copper ions are important components in the haemocyanin, and are highly regulated in all gammarid species (Taylor & Anstiss 1999). Crustaceans including the gammarid species have detoxification mechanisms to counteract toxicity by metal ions (Geffard et al. 2010). However, if these detoxification mechanisms are unable to regulate the excess of internalized metal, the excess leads to physiological disturbances (Lebrun et al. 2012). Amphipods are frequently used as bioindicators in aquatic toxicity tests owing to their prolific breeding, high abundance in nature and sensitivity to anthropogenic compounds such as metal ions in water bodies which they inhabit (e.g. Ladewig et al. 2006). *Gammarus fossarum* Koch, 1835 (Crustacea; Amphipoda) is a typical inhabitant of running waters rich in oxygen

(Lukancic et al. 2009) and abundantly inhabits springs and spring brooks in mountainous regions of central Europe (Janetzky 1994; Pöckl et al. 2003). This species is more sensitive than *Gammarus pulex* (L) towards contamination of water, low oxygen and low pH (Rinderhagen et al. 2000; Alonso et al. 2010), but is a relatively robust representative of the macrozoobenthos of springs. *G. fossarum* is mainly an efficient shredder, but also feeds on fine particulate organic matter (FPOM) (Moog 1995). It plays a fundamental role in organic matter breakdown in springs and spring brooks and hence in the distribution of coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM) (Wagner 1990; Simcic & Brancelj 2006). Furthermore it is fairly easy to keep in the laboratory. The use of a crenobiontic species for our experiments would have been more desirable; however these taxa do not occur abundantly and are therefore not suitable for experiments which require large numbers of individuals. For these reasons *G. fossarum* can be deemed a suitable organism for assessing possible impacts of Global Climatic Change and pollution.

We decided to make use of the following two endpoints in our experiments: The **feeding activity** and the respiratory Electron Transport System (**ETS**) **activity**. It is known that the metabolic activity of an organism can be expressed for example by the feeding activity and the respiratory rate. These parameters were chosen because one of them reveals information on the metabolism of the organism on the level of the organism and the other one reflects the metabolic activity of the organism on the cellular level. Furthermore the feeding and ETS activity are replicable in a fairly short time frame.

The feeding activity gives insight into the metabolic activity of the organisms on the level of the organism. Such a non-lethal endpoint is suitable for assessing low concentrations of pollutants which are found in nature (Pestana et al. 2007). The feeding activity is also a good indicator for environmental stressors (Pestana et al. 2007) such as pollution and temperature increases.

The ETS assay is a useful tool for assessing the metabolic activity of an organism on the cellular level. The ETS is an enzyme system found in the inner mitochondrial membranes of eucaryotes which controls the oxygen consumption (G.-Toth 1999). The results obtained from this

assay reflect the maximum oxygen consumption when all enzymes are functioning optimally (Kenner & Ahmed 1975).

The objective of this study was to find out how *G. fossarum* reacts to stepwise increasing water temperatures and the additional stressor copper on a sub-lethal level. This was tested in a laboratory under controlled conditions using environmentally realistic copper ion concentrations. Rising water temperatures are postulated to enhance the effects of copper pollution and generally increase the metabolic activity of *G. fossarum*. We assumed that both the feeding and ETS activity of *G. fossarum* would increase with rising temperatures, but decrease under the influence of copper ions.

Materials and Methods

Chemicals

Copper sulphate penta-hydrate ($CuSO_4*5H_2O$, Merck, Lot no. A921690 717), magnesium sulphate hepta-hydrate ($MgSO_4*7H_2O$, Merck, Lot no. A966886 729), formaldehyde solution min 37 % GR (HCHO, Merck, Lot no. K23876703 714), ortho-phosphoric acid 85 % (H_3PO_4, Merck, Lot no. K43024773 150) and potassium dihydrogen phosphate (H_2KPO_4, Merck, Lot no. K23916773 715) were purchased from Merck (Germany). Polyvinyl pyrrolidone (PVP) ($(C_6H_9NO)_x$, Sigma Aldrich, Lot no. BCBG5331V), Triton-X-100 (Triton, Sigma Aldrich, Lot no. BCBC9283V), β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH, Sigma Aldrich, Lot no. 071M7021V), β-Nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADPH, Sigma Aldrich, Lot no. SLBH3107V), 2-p-iodo-phenyl 3-p-nitrophenyl 5-phenyl tetrazolium chloride (INT, Sigma Aldrich, Lot no. BCBG6164V) and sodium phosphate dibasic dodeca-hydrate ($HNa_2O_4P*12H_2O$, Sigma Aldrich, Lot no. SZBB201AV), were obtained from Sigma Aldrich (Germany).

Collection of test organisms

G. fossarum specimens were collected from one natural spring, a rheocrene, in the Röserental near Liestal, in Switzerland (for further information see von Fumetti & Nagel (2012)), and transported

in spring water with leaves from the spring to the laboratory. Individuals of both sexes were collected and their size ranged from about 8 mm to 14 mm body length, the majority of the gammarids being about 10 mm long. The wet weight of the gammarids used in these experiments ranged from 8 to 14 mg and the selected specimens showed no form of parasitism and their movement was not impaired. They were kept at 10 °C in the transport containers for 84 hours for acclimatisation to laboratory conditions before being used in an experiment.

Conditioning of the leaf discs

As the spring the gammarids were obtained from is surrounded mainly by beech trees, these leaves form the most important food source for many of the spring inhabitants including *G. fossarum*. Therefore we collected beech leaves *(Fagus sylvatica* L.*)* from the litter layer near the spring after abscission in autumn. The collected leaves were dried in an oven at 40° C and then stored as described by Bloor (2010). Leaf discs (Ø 1 cm) were cut out of these dried leaves with the help of a cork borer and then weighed. Twelve leaf discs (more info see below) were always weighed together and then placed together in numbered stainless steel herb infusers (Ø 9 cm). The infusers were submerged into aerated spring water with FPOM from the spring for conditioning at a water temperature of ±17 °C for four weeks.

Measured physical and chemical parameters

The oxygen saturation (%) and concentration (mg / L) as well as the pH and conductivity (µS / cm) of the spring water were measured in the field using portable meters (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). The phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) concentrations (all in mg / L), of the spring water were measured with ICP-OES (SPECTRO MS, Spectro Analytical Instruments GmBh, Kleve, Germany).

Choice of copper ion concentration

In order to decide what sub-lethal concentration of copper ions to use for our experiments, we conducted LC_{50}-tests with copper sulphate and *G. fossarum* at 10, 14 and 18 °C for 96 hours.

During this time the gammarids were not fed. The copper ion concentrations for the LC_{50} testing were chosen based on values from a previous study (Güven et al. 1999). Three temperatures were tested, using 126 gammarids per temperature with 18 per tested concentration. Two controls and five nominal concentrations namely 0.05, 0.08, 0.15, 0.3and 0.5 mg Cu^{2+} / L were tested. The actual test concentrations of the copper ions in the water were not tested owing to technical and financial restrictions. The LC_{50}-values of these tests exhibited a clear pattern in that with higher temperatures less Cu^{2+} was tolerated by the gammarids; the LC_{50}-value at 10 °C after 96 hours was 0.239 mg Cu^{2+} / L, at 14 °C was 0.188 mg Cu^{2+} / L and at 18 °C was 0.135 mg Cu^{2+} / L. The LC_{10}-value at 10 °C after 96 hours was 0.164 mg Cu^{2+} / L, at 14 °C was 0.100 mg Cu^{2+} / L and at 18 °C was 0.073 mg Cu^{2+} / L. Since the main experiments were designed to last 12 days (288 hours) we decided to run a small test with 10 gammarids at 10 °C for 12 days. These gammarids were fed *ad libitum* with *Fagus sylvatica* leaf discs because 12 days of starving would have stressed and weakened the gammarids additionally. The LC_{50}-value at 10 °C after 288 hours was 0.304 mg Cu^{2+} / L and the LC_{10}-value was 0.167 mg Cu^{2+} / L (Table 1). The latter value is nearly identical to the one obtained at 10 °C after 96 hours (0.164 mg Cu^{2+} / L). The LC_{10}-value of 0.164 mg Cu^{2+} / L received after 96 hours at 10 °C was thus chosen as the sub-lethal concentration for further feeding and ETS tests. By comparison copper salt concentrations in natural unimpacted waters are mainly influenced by the geology of the water shed of the area and are in the range of < 4.00 µg / L (Schönborn & Risse-Buhl 2013). In streams natural copper ion concentrations are 4 to 12 µg / L and in groundwater it is less than 0.1 µg / L (Standard Methods for the Examination of Water and Wastewater 1998). The copper salt concentration of impacted waters can be considerably higher, and in a stream in Northern Germany for example copper ion concentrations of up to 13 mg / L have been measured (Sridhar et al. 2001).

Table 1: LC_{50}-values and confidence limits

Temperature [°C], time [hours]	LC_{50}-value [mg Cu^{2+} / L]	95% lower confidence limit [mg Cu^{2+} / L]	95% upper confidence limit [mg Cu^{2+} / L]	LC_{10}-value [mg Cu^{2+} / L]	95% lower confidence limit [mg Cu^{2+} / L]	95% upper confidence limit [mg Cu^{2+} / L]
10, 96	0.239	0.095	0.294	0.164	0.016	0.223
14, 96	0.188	0.141	0.238	0.1	0.045	0.122
18, 96	0.135	0.111	0.166	0.073	0.05	0.091
10, 288	0.304	0.226	0.412	0.167	0.074	0.224

Experimental design

The experiments were conducted in a laboratory in four stainless steel flow channels. Each unit consisted of the actual flow channel (1 m x 0.4 m x 0.2 m), a tube through which water flowed into a rain barrel (60 litres) functioning as a water reservoir, an aquarium pump (EHEIM, compact 1000, Deizisau, Germany) to pump the water into the channel and a cooling unit (Aqua Medic Titan 500, Blessendorf, Germany). The water temperature was regulated with this cooling unit with an accuracy of ± 0.5 °C. Eight plastic boxes (78 mm x 108 mm x 67 mm) were placed in every channel: four plastic boxes were filled with pure spring water (= control groups); the other four were filled with the chosen copper sulphate pentahydrate spring water solution of 0.164 mg Cu^{2+} / L (= experimental groups). Spring water from the spring from which the organisms were collected was used so that the presence of a natural microflora was given (Jonsson & Malmquist 2000). The copper ion concentration of the natural spring water was measured with ICP-EOS and found to be 0.00 mg Cu^{2+} / L. The actual test concentration of copper ions in the water of every box in the experiments was not tested owing to technical and financial restrictions. Six test organisms were placed into each box; this correlates approximately with the natural population density (Jonsson & Malmquist 2000). Organisms were used regardless of their sex, as has been done in other studies (e.g. Cold & Forbes 2004; Alonso et al. 2010; Bundschuh et al. 2009). Twelve leaf discs were placed into every box of six gammarids as the food source. Each box was aerated by a pump (Tetra, APS 50, Melle, Germany) and an air outlet stone (Trixie, Nr. 85501, Tarp, Germany), which was renewed for every experiment. This ensured constant oxygen content of the water over the entire duration of the experiments. The photoperiod was 11 hours light, 13 hours dark. All flow channels were illuminated by two different aquaria-lights (Juwel Aquarium warm-lite and Juwel Aquarium

day-lite). The experiments all lasted twelve days. During this time the temperature was varied. Channel 1 was kept at 10 °C and functioned as the **control channel** regarding the water temperature. The water temperatures in the experimental channels were increased as follows: on the first day the water temperature was set at 10 °C, on the fifth day it was raised and on the ninth day it was raised further. The temperature regime of channel 2 was 10, 12, 14 °C, that of channel 3 was 10, 14, 16 °C and that of channel 4 was 10, 16, 18 °C (Table 2). The experiments were repeated a total of 5 times to obtain 5 replicates.

Table 2: Temperature regimes applied in the conducted experiments 1-5. Channel 1: control channel, kept at 10°C during the entire experiment. Date: start of the experiments. Deaths during the experiments are given in total numbers of the 48 per channel; all deaths occurred in the copper-exposed groups and with rising water temperature more deaths occurred.

Temperature regime channels 1 - 4 days 4 – 8 – 12	Exp 1: 5/11/2012 deaths	Exp 2: 10/12 /2012 deaths	Exp 3: 7/1/2013 deaths	Exp 4: 28/1/2013 deaths	Exp 5: 25/2/2013 deaths	Total deaths per channel over all experiments
10-10-10 °C	1	1				2
10-12-14 °C	1			3		4
10-14-16 °C	1	1	1		1	5
10-16-18 °C		2	2	3		7

Feeding activity

Each flow channel held 4 boxes for feeding tests, 2 were copper-free, and 2 were with copper. The organisms were monitored daily for deaths and dead ones were removed from the boxes. At the end of the experiments all gammarids were preserved in 100% ethanol, dried at 40° C and weighed. The leaf discs they had fed on were also dried at 40°C and then weighed. Maltby et al. (2002) and many other authors using feeding activity as an endpoint for their experiments speak of feeding rates. However, considering that gammarids are shredders and hence do not necessarily eat all the leaf material they process we prefer to use the term feeding activity. We decided to determine an overall feeding activity at the end of each experiment, since it was of interest to us how the temperature elevations affected the feeding activity over the entire duration of the experiment. The

feeding activity was determined for every box, according to Maltby et al. (2002), for the 6 organisms together, as described below:

$$FA = ((Li \times \text{Control factor}) - Lf) / (\text{weight} \times \text{time})$$

where FA is the feeding activity, Li is the initial weight (mg) of the leaves, Control factor was determined experimentally by us and is the loss of weight of leaves during 12 days when no feeding takes place, Lf is the final weight (mg) of the leaves, weight is the dry weight of gammarids (mg) and time is the duration of experiment (days).

ETS activity

Each flow channel held 4 boxes for ETS tests, 2 were copper-free, and two were with copper. The ETS activity of the test organisms was determined according to the method originally developed by Packard (1971) and improved by G.-Toth (1999). All experiments started off at 10 °C; the experimental groups were then exposed to higher temperatures, while the controls were kept at 10 °C. The ETS activity was always measured 48 hours after a steady water temperature had been reached in order to detect the influence of the changing water temperatures on the organisms at a cellular level. ETS assays were carried out on days 4, 8 and 12 of the experiments. Four test organisms out of one box with copper and four from one without copper, per flow channel, were used. In total 8 test organisms per temperature and a total of 32 gammarids per day were analysed.

To conduct the ETS assay eight live gammarids were removed from the flow channels one by one using tweezers, placed on a tissue and gently patted dry. They were then weighed singly on a microbalance (XP6, METTLER TOLEDO, Greifensee, Switzerland), placed on a numbered aluminium sheet with some distilled water and then homogenised singly in the homogenisation tube without the distilled water, using 4ml of ice-cold homogenising buffer solution [0.1 M sodium phosphate buffer pH 8.4, 75 µM $MgSO_4$, 0.15% (w/v) polyvinyl pyrrolidne, 0.2% (v/v) Triton-X-100]. The homogenate was poured into a centrifuge tube and sonicated with an ultrasonic homogeniser (Bandelin Sonopuls HD2070, Berlin, Germany) for 20 seconds and stored in an ice

solution. The homogenate was then centrifuged (Sigma 2-16 PK, Osterode am Harz, Germany) at 0°C for 4 minutes at 10000 r.p.m., according to Simcic & Brancelj (2004). The supernatant (in triplicate) was incubated with 1.5 mL substrate solution [0.1 M sodium phosphate buffer pH 8.4, 1.7 mM NADH, 0.25 mM NADPH, 0.2% (v/v) Triton-X-100] and 0.5mL reagent solution [2.5 mM 2-p-iodo-phenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride] for 40 minutes at 10 °C. Stopping solution [formaldehyde (conc.):H_3PO_4 (conc.) = 1:1], 0.5mL, was added immediately after incubation and the formazan production determined spectrophotometrically with the spectroquant ® Pharo 300 (Merck, Darmstadt, Germany) by measuring the absorbance of the sample at 490nm against the blank.

In order to obtain a conversion to equivalent oxygen the ETS activity was calculated according to Kenner & Ahmed (1975) as follows:

$$\text{ETS activity } (\mu L\ O_2 / mg \times h) = ((Abs^{490nm} \times Vr \times Vh \times 60) / (Va \times Gw \times t \times 1.42)$$

where Abs^{490nm} is the absorption of the sample, Vr is the final volume of the reaction mixture (3mL), Vh is the volume of the original homogenate (4mL), Va is the volume of the aliquot of the homogenate (0.5mL), Gw is the gammarid wet weight (mg), t is the incubation time (minutes) and 1.42 is the factor for conversion to volume oxygen.

Data analysis

The programme “EPA Probit Analysis Program, Version 1.5” (http://www.epa.gov/) was used to determine LC_{50}- and LC_{10}-values of *G. fossarum* specimens for copper sulphate. The programme is based on the method of probit analysis, which is a type of regression used to analyse binomial response variables, in this case concentration and mortality of the test individuals, always at the different temperatures. The method transforms the sigmoid dose-response curve to a straight line (Newmann, 2010). This line may be fitted by the method of weighted least squares (Litchfield & Wilcoxon, 1948).

The effects of the copper ion concentration and water temperatures on *G. fossarum* were tested using one and two way ANOVAs followed by Scheffes's post-hoc tests. Prior to the ANOVA analyses assumptions of variance homocedasticity and data normality were tested using Levene's and Kolgomorof-Smirnov's tests. To correct for multiple single comparisons a Bonferroni-Holm correction was applied. The α-level was set at 0.05. Analyses were performed using the Statistical Package for Social Sciences (SPSS) version 21 for Windows (SPSS Inc, Chicago, IL, U.S.A.)

Results

Measured physical and chemical parameters

The temperature of the spring water ranged from 10 to 11 °C; the pH ranged from 6.9 to 7.4; the electrical conductivity of the spring water ranged from 534 µS / cm to 684 µS / cm; the oxygen concentration was between 6.8 mg / L and 11.8 mg / L and the saturation between 65 % and 98 %; the phosphate (PO_4^{3-}) and the nitrite (NO_2^-) concentrations were < 0.05 mg / L; the ammonia (NH_4^+) concentration of the spring water was < 0.1 mg / L and the nitrate (NO_3^-) concentration was 18.0 mg / L.

Feeding activity

The feeding activity ranged from 0.008 to 0.373 mg / mg × d. The lowest value was measured in a copper-exposed box in the control channel at 10°C and the highest in a copper-free box in an experimental group with the temperature regime 10-12-14 °C. A tendency towards higher feeding activity at the intermediate temperature regime 10-12-14 °C in the control group can be noted (Fig. 1).

A two-way ANOVA showed that the copper ions did not have a significant effect on the feeding activity, $F(1, 72) = 1.5$, $p = 0.225$ and that temperature had a nearly significant effect, $F(3, 72) = 2.612$, $p = 0.058$. The interaction of temperature and copper ions was not significant, $F(3, 72) = 0.807$, $p = 0.494$.

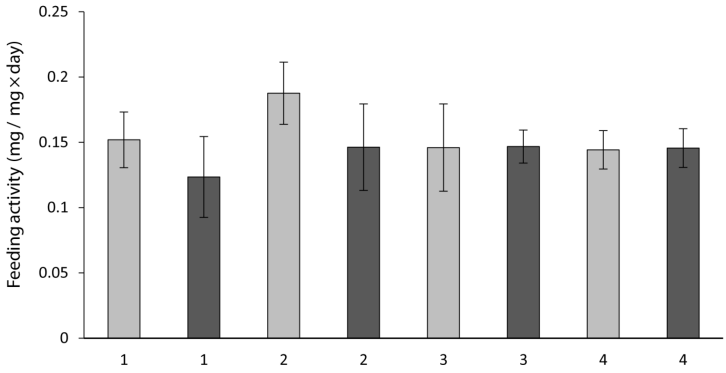


Fig. 1 Calculated mean feeding activityof *G. fossarum* at the different conditions, with and without copper ion influence. The control channel (1) is always at 10 °C, the experimental channels (2, 3 and 4) have temperature regimes which range from 10 ° to maximum 18 °C. Standard errors are represented by the error bars attached to each column. Pale grey: no copper, Dark grey: copper influence.

ETS activity

The ETS values ranged from 0.174 to 1.431µL O_2 / mg × h. The lowest value was measured in a gammarid at 10 °C exposed to copper ions and the highest in a gammarid at 10 °C not exposed to copper ions. The ETS values of the gammarids not exposed to copper ions were all higher than the ETS activities of the gammarids exposed to copper ions (Fig. 2).

A two-way ANOVA showed that the copper ions had a highly significant effect on the ETS activities of the gammarids $F(1, 463) = 19.625$, $p < 0.001$. The temperature also had a significant influence on the ETS activities $F(4, 463) = 3.683$, $p = 0.006$. The interaction of temperature and copper ions was not significant $F(4, 463) = 0.527$, $p = 0.716$.

A one-way ANOVA comparing the different treatments (copper-free & copper-exposed) in the control channel, channels 2, 3 and 4 showed significant ($p = 0.002$; $p < 0.001$; $p = 0.030$; $p = 0.028$, respectively) effects of **copper** ($F_{1,121} = 9.558$; $F_{1,118} = 14.435$; $F_{1,108} = 4.845$; $F_{1,102} = 4.939$, respectively). In all channels copper reduced the ETS activity (Fig. 2).

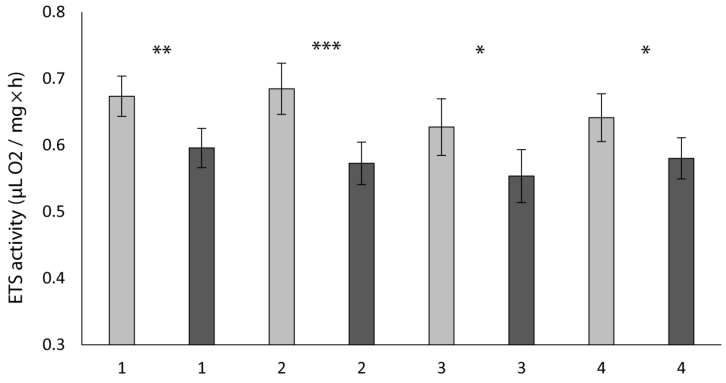
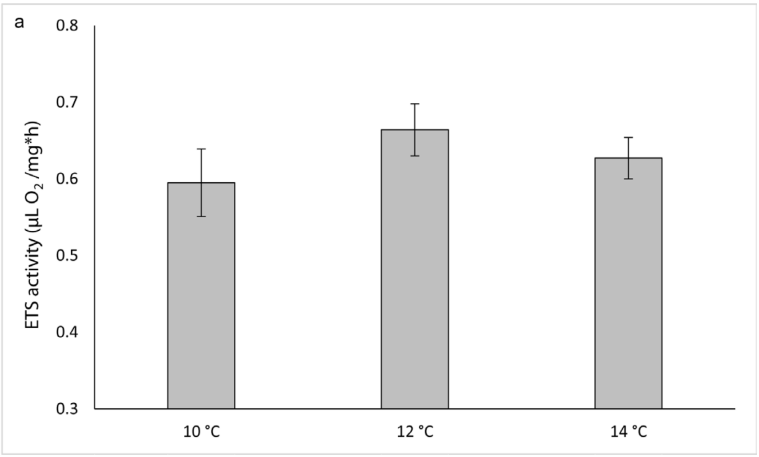


Fig. 2 Mean ETS activities of *G. fossarum* for all channels with their different temperature regimes, with and without copper ion influence. The control channel (1) is always at 10 °C, the experimental channels (2, 3 and 4) have temperature regimes which range from 10 ° to maximum 18 °C. Standard errors are represented by the error bars attached to each column. Pale grey: no copper, Dark grey: copper influence. Note: *** < 0.001, ** < 0.003, * < 0.05

Temperature did not affect the ETS activity in channel 2 ($F_{2,118} = 1.75$) significantly ($p = 0.178$), but did so significantly ($p = 0.008$; $p < 0.001$, respectively) in channels 3 and 4 ($F_{2,108} = 5.017$; $F_{2,102} = 9.903$, respectively) increasing the ETS activity (Fig. 3a-c).



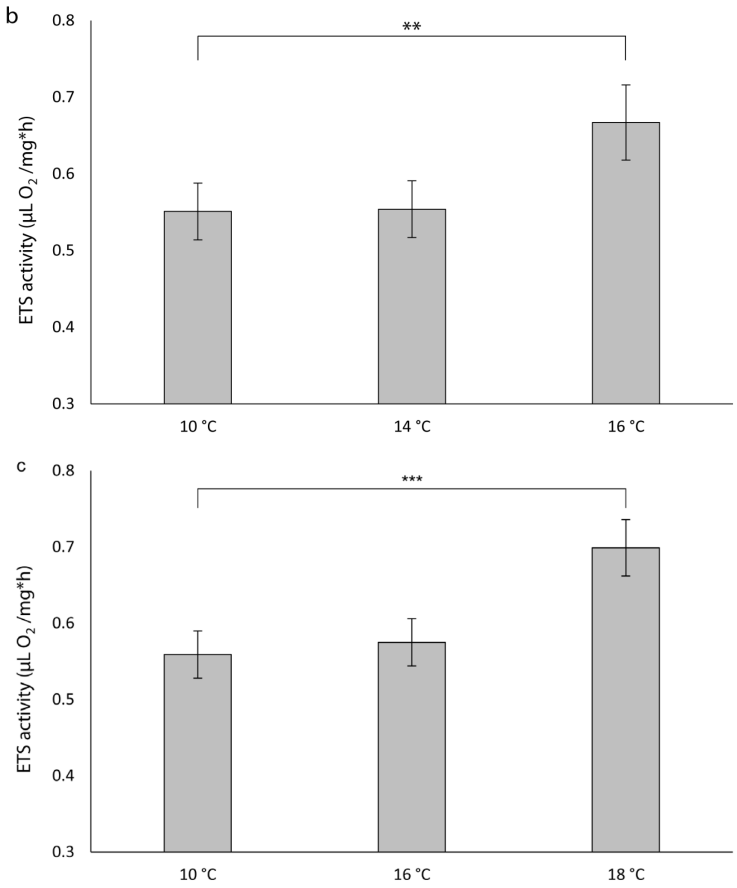


Fig. 3a-c Mean ETS activities of *G. fossarum* for the channels 2(a), 3(b) and 4(c) with their different temperature regimes, regardless of the copper ion influence. Standard errors are represented by the error bars attached to each column. Note: *** < 0.001, ** < 0.003, * < 0.05

Discussion

It is known that heavy metals affect the metabolic activity of organisms, their behavior and their distribution in the ecosystems and that temperature is a very important factor influencing this as well (Lemus & Chung 1999). Temperature has often been quoted to have an effect on the toxicity of pesticides and other pollutants (Fent 2013) and it has been shown that increasing temperatures increase the toxicity of copper (Holmstrup et al. 2010). We therefore assumed that higher water temperatures would significantly change the susceptibility of the tested organisms to copper.

In our experiments the feeding activity was not significantly changed by the sub-lethal copper ion concentration or higher water temperatures; however a trend towards higher feeding activity at higher water temperatures could be seen. The ETS activity of *G. fossarum* increased significantly at

elevated temperatures and the presence of copper ions significantly lowered the ETS activity. Our findings demonstrate the benefit of using two different approaches when examining environmental changes on an organism.

Feeding activity

The chosen tree species can affect the feeding activity considerably and most feeding tests described in literature are conducted with *Alnus glutinosa* leaves (e.g. Cold & Forbes 2004; Felten et al. 2008; Bundschuh et al. 2009), these apparently being the preferred leaf species of *G. fossarum* and other detritivores (Haeckel et al. 1973; Naylor et al. 1990; Bloor 2009). We chose *Fagus sylvatica* as the food source because the springs from which the gammarids for this study were collected are surrounded by beech trees and we aimed to provide the gammarids with their natural food source. The feeding activities measured in this study are similar, albeit a bit lower, than those recorded by Maltby et al. (2002) with *G. pulex*. The feeding activity of our control group was however higher than the one recorded by Felten et al. (2008). In both cited studies alder leaves were used. The results of our study suggest that well-conditioned beech leaves are also a palatable food source for gammarids.

Feeding tests conducted with *G. pulex* at different temperatures showed that the consumption rose with temperature (Nilsson 1974). In an in-situ study *G. pulex* had a lower feeding activity in April compared to August (Bloor & Banks, 2006). The authors suggest that this was owing to temperature stress: in April it was too cold and hence the organisms ate less. The gammarids used in our experiments were all collected in winter between November and March and so we do not expect any seasonal effects. In our study the higher water temperatures had nearly significant effects on the feeding activity, both in the copper-free and copper-exposed groups. Significance should be reached by longer test periods. In tests lasting 26 days we were able to show that increasing water temperatures increased feeding activity of *G. fossarum* significantly (Schmidlin et al. subm.). Coulaud et al. (2011) also found an increase of the feeding activity at elevated water temperatures for *G. fossarum*. The same has been shown for *G. pulex* by Maltby et al. (2002). A distinctly higher feeding activity of the gammarids not exposed to copper in the

condition 10 – 12 – 14 °C was observed. This temperature range is actually optimal for *G. fossarum*, as it has been shown that the ideal temperature for optimal reproduction lies at 12 °C (Pöckl & Humpesh 1990).

In the control condition and condition 10-12-14 °C there was a slight trend towards lower feeding activity of the gammarids exposed to copper ions compared to the gammarids not exposed to copper ions. Previously a decreasing feeding rate of *G. pulex* when exposed to copper was measured (Taylor et al. 1993). The concentrations they used were between 0.01 and 0.150 mg / L Cu^{2+} and the highest one being nearly identical to the one used here. Their findings support our observation. It has been shown previously that the feeding rate of *G. pulex* decreased with increased cadmium ion concentrations (Felten et al. 2008; Alonso et al. 2009). Brief exposure of *G. pulex* to high concentrations of Lindane has been shown to have the largest effect on the feeding rate of *G. pulex* during the first 24 hours after exposure (Malbouisson et al. 1995). The feeding activities in our study may have been lower after the first few hours of exposure and then, owing to adaption, rise slightly to a constant level. We cannot be sure of this because we determined a feeding activity for the entire duration of the experiment.

We assumed that higher temperatures would enhance the negative effects of copper ion exposure and so reduce the feeding activity. Furthermore we assumed that elevated temperatures would cause higher metabolic activity and so an increase in feeding activity would take place. Higher temperatures did not cause a clear increase in the feeding activity of *G. fossarum* in this study and the copper ions did not significantly reduce the feeding activity. A reason for the copper ions not having a significant effect on the feeding activity is most likely because a sub-lethal copper ion concentration was chosen for these tests. A further reason may be that partial adsorption of the copper ions to the leaf discs (Tattersfield 1993) reduced the copper ion concentration in the water and this in turn might explain why the feeding activity at the higher temperatures was not significantly higher. The average water temperatures of the four channels (10 °C, 12 °C, 13.3 °C and 14.7 °C) are not as different from each other as the temperature regimes might suggest, but the temperature jumps are far more important, since these doubtlessly affected the gammarids. The temperature jumps in channels 3 and 4 are extreme. We speculate that the gammarids had to first

become accustomed to the much higher temperature (i.e. from 10 °C to 14 °C and 10 °C to 16 °C) before they resumed feeding which resulted in an overall similar feeding activity to the control 10- 10-10 °C. The feeding activity of *G. fossarum* was highest in the channel 2, where the jumps of 2 °C from 10 °C to 12 °C and then to 14 °C effectively made the conditions for *G. fossarum* more optimal.

ETS activity

Our ETS results show that a change on the cellular level of the organisms was caused both by increased temperature and copper ion exposure. In two previously published studies the ETS activities for *G. fossarum* ranged from 0.4 to 0.48 µL O_2 / mg × h and from 0.39 to 0.45 µL O_2 / mg × h (Lukancic et al. 2010; Simcic 2005). During fasting the ETS activity of *G. fossarum* has also been found to be in a similar range (0.36 to 0.58 µL O_2 / mg × h) (Mezek et al. 2010). These activities are just slightly lower than the ones recorded in our study.

With rising water temperatures we measured a significant increase of the ETS activity of *G. fossarum* within the copper-free and copper-exposed groups in channels three and four. An increase in ETS activity with increasing water temperatures has also been observed e.g. for daphnid species and hybrids: both juveniles and adults displayed a higher ETS activity at higher temperatures (Simcic & Brancelj 1997; Simcic & Brancelj 2004). The absolute ETS activities of channels two, three and four are lower than those of the control channel at the same point in time. This can be explained by two competing factors: with higher temperatures the metabolism of the organisms is more efficient, on the other hand the oxygen concentration is lower the higher the temperature becomes.

G. fossarum exposed to copper showed significant decreases in ETS activity compared to those in pure spring water. It has been shown that a copper ion concentration of 10 µg Cu^{2+} / L caused a small but significant increase in the ETS activity of mixed zooplankton while the lower concentration of 5µg Cu^{2+} / L had no significant effect (Bamsted 1980). On the other hand it has been found that Atrazine and Imidacloprid reduced the ETS activity of *G. fossarum* (Lukancic et al. 2010). Bamsted's observation of an increase in ETS activity emphasizes that different taxa react

differently to metal pollution. It has also been demonstrated that cadmium and chromium affected the ETS activity of *Daphnia magna* differently: cadmium did not affect cellular respiration after 48 hours of exposure; chromium however caused an increase after 48 hours and a decrease after 96 hours of exposure in the ETS activity (De Coen & Janssen 2003). This is similar to the ETS activities we measured in the copper-exposed gammarids at the temperature regimes 10 to 14 °C and 10 to 16 °C, where the ETS activity did not increase continuously but fluctuated. Many enzymes are organometallic compounds (Meyer 2001) and hence one would expect heavy metals to have some effect on the enzyme activity which would reflect in the ETS activity.

Implications for springs

The results of this study show that the exposure to Cu^{2+} ions coupled with a higher temperature has the potential to be stressful to *G. fossarum*. A temperature elevation on its own, however, is unlikely to be very problematic for eurythermal species as can be seen from our results. In a study by Pöckl et al. (2003) it was concluded that a temperature rise in rivers with a current mean temperature of 7 to 10 °C would not affect *G. fossarum* and *G. roeseli* greatly, but a warming of colder rivers would have a positive effect for the gammarids and that a warming of already warm flowing waters would be negative. His study underpins our assumption that *G. fossarum* is fairly tolerant towards slightly elevated temperatures, with the clear understanding that these do however influence their life history, especially when coupled with pesticides and other pollutants, and that larger temperature elevations of more than 4 °C are problematic. However, *G. fossarum* has a narrower distribution than *G. pulex* because it is more sensitive to environmental variables and is also often more sensitive towards pollutants (Alonso et al. 2010). *G. fossarum* probably also does not occur in warmer waters because *G. pulex* is the stronger of the two competitors, being more robust towards higher water temperatures up to 27 °C (Foucreau et al., 2014; Sutcliffe et al., 1981) and so *G. fossarum* is forced into colder waters.

Assuming Global Change raises temperatures further this might nevertheless bring the gammarids into difficulties, because raised metabolisms and hence higher feeding activity could cause food to become a limiting factor, because allochthonous in-put of leaf litter is finite.

Although springs are known for their clean water, pollution of these unprotected habitats in Switzerland (Zollhöfer, 1997) can occur. Our study demonstrates the importance of clean spring water for organisms, especially when an increase in water temperatures cannot be prevented. The findings emphasize the need for springs and headwaters and their species to be protected from pesticide inputs and other forms of pollution and to be monitored regularly. We worked with *G. fossarum* in this study as an abundant representative of spring species and have been able to show that a gradual temperature elevation will probably not pose a problem for *G. fossarum* and other eurythermal species. *G. fossarum* nevertheless reacted when exposed to copper ion and elevated temperatures. It can be deduced that exposure to a pollutant will be tolerated even worse by cold-stenothermal organisms and they will most probably react much more sensitively to elevated temperatures. It has been shown that the respiratory ETS activity in cold-stenothermal and eurythermal chironomid larvae from high mountain lakes increased with rising temperatures for two chironomid genera (Simcic et al. 2005). Different responses to temperature changes were observed between cold-stenothermal and eurythermal genera, especially at high temperatures. The findings of Simcic et al. (2005) are in accordance with the assumption that cold-stenothermal spring species would react even more sensitively to temperature changes. While the ability of survival of many species may decrease, a few species might find more ecological niches to live in and hence their populations might increase. A consequence of environmental pollution and changes will, however, presumably be an overall loss of biodiversity in springs.

Conclusions

Higher water temperatures increase the metabolic activity of *G.fossarum* and copper ions have a tendency to decrease the feeding activity and significantly decrease ETS activity of the tested organisms. *G. fossarum* can cope with a slight elevation in temperature; copper however impairs its general fitness. The ETS assay has proved to be more sensitive in detecting the effects of sub-lethal copper ion concentrations on the metabolic activity of *G. fossarum* than the feeding tests. Therefore we propose the usage of the ETS assay in addition to the well-established feeding tests for more

detailed results. Further experiments will be conducted with a cold-stenothermal species in order to test its responses to a temperature increase and exposure to copper.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Alonso A, De Lange HJ, Peeters ETHM (2009) Development of a feeding behavioural bioassay using the freshwater amphipod *Gammarus pulex* and the Multispecies Freshwater Biomonitor. Chemosphere 75: 341-346.

Alonso A, De Lange HJ, Peeters ETHM (2010) Contrasting sensitivities to toxicants of the freshwater amphipods *Gammarus pulex* and *G. fossarum*. Ecotoxicol 19: 133-140.

Bamsted U (1980) ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. J of Experimental Mar Biol and Ecology 42: 267-283.

Bat L, Akbulut M, Culha M, Gündogdu A, Satilmis HH (2000). Effect of Temperature on the Toxicity of Zinc, Copper and Lead to the Freshwater Amphipod *Gammarus pulex pulex* (L., 1758). Turk J Zool: 24: 409-415.

Bloor M (2009) Aquatic Pollution. Case Study of Landfill Leachate Toxicity and Remediation. VDM Verlag.

Bloor MC (2010) Animal standardisation for mixed species ecotoxicological studies: Establishing a laboratory breeding programme for *Gammarus pulex* and *Asellus aquaticus*. Zoologica baetica 21: 179-190.

Bloor MC, Banks CJ (2006) An evaluation of mixed species in-situ and ex-situ feeding assays: The altered response of *Asellus aquaticus* and *Gammarus pulex*. Environ Internatl 32: 22 – 27.

Brooks SJ, Mills CL (2003) The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comparative Biochemistry and Physiol Part A 135: 527-537.

Bundschuh M, Hahn T, Gessner MO, Schulz R (2009) Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. Environ Toxicol and Chem 28: 197-203.

Cold A, Forbes VE (2004) Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. Aquat Toxicol 67: 287-299.

Coulaud R, Geffard O, Xuereb B, Lacaze E, Quéau H, Garric J, Charles S, Chaumot A (2011) In situ feeding assay with *Gammarus fosssarum* (Crustacea): Modelling the influence of confounding factors to improve water quality biomonitoring. Water Res 45: 6417-6429

Danks HV, Williams DD (1991) Arthropods of springs, with particular reference to Canada: Synthesis and needs for research. In Williams, D. D. & H. V. Danks (eds), Arthropods of springs, with particular reference to Canada. Memoirs of the Entomological Soc of Can 155: 203–217.

Debelius B, Forja JM, DelValls A, Lubian L(2009)Toxicity and bioaccumulation of copper and lead in five marine microalgae. Ecotoxicol and Environ Saf 72: 1503-1513.

Dedourge-Geffard O, Palais F, Biagianti-Risbourg S, Geffard O, Geffard A (2009) Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: An *in situ* experiment. Chemosphere 77: 1569-1576.

De Coen WM, Janssen CR (2003) The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of the toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ Toxicol and Chem 22: 1632-1641.

De Martinez Gaspar Martins M, Barcarolli IF, De Menezes EJ, Giacimin MM, Wood CM, Bianchini A (2011) Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: *In vivo* and *in vitro* studies. Aquat Toxicol 101: 88-99.

De Oliveira-Filho EC, Lopes RM, Paumgartten FJR (2004) Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56: 369-374.

Effler SW, Litten S, Field SD, Tong-Ngork T, Hale F, Meyer M, Quirk M (1980) Whole lake responses to low level copper sulphate treatment. Water Res 14: 1489-1499.

Felten V, Charmantier G, Mons R, Geffard A, Rousselle P, Coquery M, Garric J, Geffard O (2008) Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquat Toxicol 86: 413-425.

Fent K (2013) Ökotoxikologie. Fourth Edition. Thieme Verl., Stuttgart, New York.

Ferrington LC (1995) Biodiversity of aquatic insects and other invertebrates in springs: Introduction. J of the Kans Entomological Soc. 68: 1-3.

Fischer J, Fischer F, Schnabel S, Bohle HW (1998) Spring fauna of the Hessian Mittelgebirge: Population structure, adaptative strategies, and relations to habitats of the macroinvertebrates, as exemplified by springs in the Rhenisch metamorphic shieldand in the East-Hessian sandstone plate. In: Botosaneanu, L. (ed.), Studies in crenobiology. The Biology of Springs and Springbrooks. Backhuys Publishers, Leiden: 182-199.

Foucreau N, Cottin D, Piscart C, Hervant F (2014) Physiological and metabolic responses to rising temperature in Gammarus pulex (Crustacea) populations living under continental or Mediterranean climates. Comparative Biochem and Physiol Part A 168: 69-75

Geffard A, Sartelet H, Garric J, Biagianti-Risbourg S, Delahaut L, Geffard O (2010) Subcellular compartmentalization of cadmium, nickel, and lead in *Gammarus fossarum*: comparison of methods. Chemosphere 78:822-829.

G.-Toth L (1999) Aktivität des Elektronentransportsystems. In: Biologische Gewässeruntersuchung. Methoden der Biologischen Wasseruntersuchung 2 (Eds W. von Tümpling & G. Friedrich). Gustav Fischer Verl., Jena, Stuttgart, Lübeck, Ulm, pp 465-473

Guida M, Inglese M, Meric S(2008)A multi-battery toxicity investigation on fungicides. Desalination 226: 262-270.

Güven K, Özbay C, Ünlü E, Satar A (1999) Acute Lethal Toxicity and Accumulation of Copper in *Gammarus pulex* (L.) (Amphipoda). Turkish J of Biol 23: 513-521.

Haeckel JW, Meijering MPD, Rusetzki H (1973) *Gammarus fossarum* Koch als Fallaubzersetzer in Waldbächen. Freshw Biol 3: 241-249.

Heino J, Virkkala R, Toivonen H (2009) Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. Biological Rev 84: 39-54.

Holmstrup M, Bindesbøl A-M, Oostingh GJ, Duschl A, Scheil V, Köhler H-R, Loureiro S, Soares AMVM, Ferreira ALG, Kienle C, Gerhardt A, Laskowski R, Kramarz PE, Bayley M, Svendsen C, Spurgeon DJ (2010) Interactions between effects of environmental chemicals and natural stressors: A review. Sci of the Total Environ 408: 3746-3762

IPCC (2007) Climate Change, 2007. Synthesis Report. Contribution of working Groups 1, 2 and 3 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. (Eds. Pachauri, R. K. & Reisinger, A.). IPCC, Geneva, Switzerland.

Janetzky W (1994) Distribution of the genus *Gammarus* (Amphipoda: Gammaridae) in the River Hunte and its tributaries (Lower Saxony, northern Germany). Hydrobiologia 294: 23-34

Jonsson M, Malmquist B (2000) Ecosystem process rate increases with animal species richness: evidence from leaf-eating, aquatic insects. OIKOS 89: 519-523.

Kenner RA, Ahmed SI (1975) Measurements of electron transport activities in marine phytoplancton. Mar Biol 33:119-127.

Komarek M, Cadkova E, Chrastny V, Bordas F, Bollinger JC (2010) Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. Environ Internatl 36: 138-151.

Ladewig V, Jungmann D, Köhler H-R, Schirling M, Triebskorn R, Nagel R (2006) Population Structure and Dynamics of *Gammarus fossarum* (Amphipoda) Upstream and Downstream from Effluents of Sewage Treatment Plants. Archives of Environ Contam and Toxicol 50: 370-383.

Lebrun J.D, Perret M, Geffard A, Gourlay-Francé C (2012) Modelling copper bioaccumulation in *Gammarus pulex* and alterations of digestive metabolism. Ecotoxicology 21: 20022-2030.

Lemus MJ, Chung KS (1999) Effect of Temperature on Copper Toxicity, Accumulation and Purification in Tropical Fish Juveniles *Petenia Kraussii* (Pisces: Cichlidae). Caribb J of Sci 35: 64-69.

Lindegaard C, Brodersen KB, Wiberg-Larsen P, Skriver J (1998) Multivariate analyses of macrofaunal communities in Danish springs and springbrooks. Studies in Crenobiology. The

Biology of Springs and Springbrooks (Ed. L.Botosaneanu), 201-219. Backhuys Publishers, Leiden, the Netherlands.

Litchfield JT Jr, Wilcoxon F (1948) A simplified method of evaluating dose-effect experiments. Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut.

Lukancic S, Zibrat U, Mezek T, Jerebic A, Simcic T, Brancelj A (2009) Effects of exposing two non-target crustacean species, *Asellus aquaticus* L., and *Gammarus fossarum* Koch, to Atrazine and Imidacloprid. Bull of Environ Contam and Toxicol 84: 85-90.

Lukancic S, Zibrat U, Mezek T, Jerebic A, Simcic T, Brancelj A (2010) A new method for early assessment of effects of exposing two non-target crustacean species, *Asellus aquaticus* and *Gammarus fossarum*, to pesticides, a laboratory study. Toxicol and Industrial Health 26: 217-228.

Malbouisson JFC, Young TWK, Bark AW (1995) Use of feeding rate and repairing of precopulatory *Gammarus pulex* to assess toxicity of Gamma-Hexachlorocyclohexane (Lindane). Chemosphere 30: 1573-1583.

Maltby L, Clayton SA, Wood RM, McLoughlin N (2002) Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. Environ Toxicol and Chem 21: 361-368.

Meyer F (2001) Wie die Natur Chemie betreibt. Universität Heidelberg. Ruperto Carola 3.

Mezek T, Simcic T, Arts MT, Brancelj A (2010) Effect of fasting on hypogean (*Niphargus stygius*) and epigean (*Gammarus fossarum*) amphipods: a laboratory study. Aquat Ecology 44: 397-408.

Moog O (1995)Fauna Aquatica Austriaca. Wasserwirtschaftskataster, Bundesministerium für Land- und Forstwirtschaft, Wien.

Naylor C, Maltby L, Calow P (1990) Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. Hydrobiologia 188/189: 517-523.

Newmann MC (2010) Fundamentals of Ecotoxicology. Third Edition. CRC Press, Taylor & Francis Group.

Nilsson LM (1974) Energy Budget of a Laboratory Population of *Gammarus pulex* (Amphipoda). OIKOS 25: 35-42.

Packard TT (1971) The measurement of respiratory electron transport system activity in marine phytoplankton. J of Mar Res 29: 235-244.

Pestana JLT, Re A, Nogueira AJA, Soares AMVM (2007) Effects of Cadmium and Zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68: 1556-1562.

Pöckl M, Humpesh UH (1990) Intra- and inter-specific variations in egg survival and brood development time for Austrian populations of *Gammarus fossarum* and *G. roeseli* (Crustacea: Amphipoda). Freshw Biol 23: 441-455.

Pöckl M, Webb BW, Sutcliffe DW (2003) Life history and reproductive capacity of *Gammarus fossarum* and *G. roeseli* (Crustacea: Amphipoda) under naturally fluctuating water temperatures: a simulation study. Freshw Biol 48: 53-66.

Reichmuth JM, Weis P, Weis JS (2010) Bioaccumulation and depuration of metals in blue crabs (*Callinectes sapidus* Rathbun) from contaminated and clean estuary. Environ Pollut 158: 361-368

Rinderhagen M, Ritterhof J, Zauke G (2000) Crustaceans as Bioindicators. From: Biomonitoring of Polluted Water - Reviews on Actual Topics (A. Gerhardt, ed.), Trans Tech Publications - Scitech Publications, Environ Res Forum 9: 161-194.

Ruyters S, Salaets P, Oorts K, Smolders E (2013) Copper toxicity in soils under established vineyards in Europe: A survey. Sci of Total Environ 443: 470-477.

Schönborn W, Risse-Buhl U (2013) Lehrbuch der Limnologie. 2. Vollständig überarbeitet Auflage. Schweizerbart, Stuttgart, p 471

Simcic T (2005) Respiratory electron transport system (ETS) activity and respiration rate in cold-stenothermal and eurythermal chironomid larvae from high-mountain lakes. Archiv für Hydrobiologie 162: 399-415.

Simcic T, Brancelj A (1997) Electron transport system (ETS) activity and respiration rate in five *Daphnia* species at different temperatures. Hydrobiologia 360: 117-125.

Simcic T, Brancelj A (2004) Respiratory electron transport system (ETS) activity as an estimator of the thermal tolerance of two *Daphnia* hybrids. J of Plankton Res 26: 525-534.

Simcic T, Brancelj A (2006) Effects of pH on electron transport system (ETS) activityand oxygen consumption in *Gammarus fossarum*, *Asellus aquaticus* and *Niphargus sphagnicolus*. Freshw Biol 51: 686-694.

Simcic T, Lukancic S, Brancelj A (2005) Comparative study of electron transport system activity and oxygen consumption of amphipods from caves and surface habitats. Freshw Biol 50: 494-501.

Sridhar KR, Krauss G, Bärlocher F, Raviraja NS, Wennrich R, Baumbach R, Krauss G-J (2001) Decomposition of alder leaves in two heavy metal-polluted streams in central Germany. Aquat Microb Ecol 26: 73-80

Standard Methods for the Examination of Water and Wastewater, 20th Edition, 1998.

Sutcliffe DW, Carrick TR, Willoughby LG (1981) Effects of diet, body size, age and temperature on growth rates in the amphipod *Gammarus pulex*. Freshw Biol 11: 183-214.

Taylor HH, Anstiss JM (1999) Copper and haemocyanin dynamics in aquatic invertebrates. Mar and Freshw Res 50:8: 907-931.

Taylor EJ, Jones DPW, Maund SJ, Pascoe D (1993) A New Method for Measuring the Feeding Activity of *Gammarus Pulex* (L.). Chemosphere 26: 1375-1381.

Taylor EJ, Underhill KM, Blockwell SJ, Pascoe D (1998)Haem biosynthesis in the freshwater macroinvertebrate *Gammarus pulex* (L.): Effects of copper and Lindane. Water Res 32: 2202-2204.

Tattersfield LJ (1993) Effects of copper on the energy budget of a stream detritivore: validation and ecological relevance. Dissertation. University of Sheffield, Sheffield.

Von Fumetti S, Nagel P (2012) Discharge variability and its effect on faunistic assemblages in springs. Freshw Sci 31: 647-656.

Vittoz P, Cherix D, Gonseth Y, Lubini V, Maggini R, Zbinden N, Zumbach S (2013) Climate change impacts on biodiversity in Switzerland: A review, J of Nat Conservation 21: 154-162

Wagner R (1990) Influence of Temperature, Photoperiod and Nutrition on Growth and Consumption of Chaetopteryx Villosa (Trichoptera). Holacrtic Ecology 13: 3: 247-254

Webb DW, Wetzel MJ, Reed PC, Philippe LR, Young TC (1998)The macroinvertebrate biodiversity, water quality, and hydrogeology of ten karst springs in the Salem Plateau of Illinois. In: Botosaneanu, L. (ed.), Studies in crenobiology: the biology of springs and springbrooks. Backhuys Publishers, Leiden: 39-48.

Woodward G, Friberg N, Hildrew AG (2009) The need for scientific rigour in biomonitoring and conservation of fresh waters. Freshwater ecosystems: biodiversity, management and conservation. Hauptpage, NY: Nova.

Woodward G, Perkins DM, Brown LE (2010) Climate change and freshwater ecosystems; impacts across multiple levels of organization. Philosophical Transactions of the R Soc B 365: 2093-2106.

Zollhöfer JM (1997) Quellen - die unbekannten Biotope im Schweizer Jura und Mittelland. Erfassen -Bewerten - Schützen. Bristol Stiftung, Zürich.

CHAPTER FOUR

Copper Sulphate Reduces the Metabolic Activity of *Gammarus fossarum* in Laboratory and Field Experiments

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Abstract
The specialised fauna of freshwater springs is affected by contamination of the water with xenobiotics from human activities in the surrounding landscape. We assessed the effects of exposure to toxins in laboratory and field experiments by using copper sulphate as a model substance and *Gammarus fossarum* Koch, 1836, as the model organism. This amphipod is a common representative of the European spring fauna and copper is a widespread contaminant, mainly from agricultural practice. The experiments were conducted in test chambers placed in flow channels and directly in a spring. The gammarids were fed with conditioned beech leaf discs, which had been exposed to a 0.8 mg Cu / L solution for 96 hours. The feeding activity of the amphipods was quantified on the level of the organism; and the respiratory electron transport system (ETS) assay was conducted in order to determine changes on the cellular level in the test organisms. The results show that the feeding activity when the leaf discs were contaminated with copper was not significantly different from the control. The ETS activity of the gammarids which had been feeding on the copper contaminated leaf discs was however significantly reduced. The results followed the same pattern for gammarids from both the laboratory and the spring. By conducting the experiments not only in a laboratory but also directly in a spring in the field, we took a crucial step towards a more realistic approach when examining environmental pollutants on an organism. Our findings demonstrate the importance of conducting experiments out in the field, in natural conditions, as well as in the laboratory.

Keywords
Amphipod, feeding activity, electron transport system (ETS), metal, accumulation, spring, headwater

1. Introduction

Pollution of our freshwaters is taking place rapidly, for example through pharmaceutical compounds, fertilisers and pesticides. Terrestrially applied pesticides are flushed into springs and rivers through runoff. This will take place more regularly when heavy rainfalls occur more frequently as a consequence of Global Change which is causing an increase in extreme events such as floods in the temperate regions (IPCC, 2007). An increase of the mean winter precipitation in the northern and western part of Switzerland and an increase of heavy precipitation events is documented for Switzerland (Schmidli et al., 2002; Schmidli & Frei, 2005).

Copper salts are important ingredients in many fungicides and fertilisers used in agriculture (e.g. de Oliveira-Filho et al., 2004) for example in vineyards (e.g. Ruyters et al., 2013) and are one of the most widespread contaminants (Debelius et al., 2009). Contamination of leaf litter with copper can happen, for example, in vineyards where fungicides containing copper are applied. Owing to its non-degradability, copper moves up food webs and is distributed in the entire biotic compartment of freshwaters (Lebrun et al., 2012). Copper concentrations in natural unimpacted waters are mainly influenced by the geology of the watershed of the area and are typically less than 4 µg / L (Schönborn & Risse-Buhl, 2013). The copper concentration of impacted waters can be considerably higher, reaching more than 10 mg / L (e.g. Sridhar et al., 2001). Although a certain amount of copper is essential for most organisms, it is extremely toxic for aquatic organisms beyond certain threshold levels (Prato et al., 2013). Copper poses a threat to many aquatic organisms when available in excess in water (De Martinez Gaspar Martins et al., 2011). A consequence of exposure to copper salts is the accumulation of these ions in the tissues of the exposed organisms. Bioaccumulation of copper has been observed in different aquatic species (e.g. Tattersfield, 1993; Reichmuth et al., 2010; Pinho et al., 2011).

Amphipods are frequently used as bioindicators in aquatic toxicity tests owing to their prolific breeding, high abundance in nature and sensitivity to anthropogenic compounds (e.g. Ladewig et al., 2006) such as metal ions in water bodies which they inhabit. Amphipods mainly take up ions via their gills since these are a large adsorptive organ system (Reichmuth et al., 2010) making them especially susceptible to water-borne pollutants (Rinderhagen et al., 2000).

The genus *Gammarus* is most commonly used in experiments in Europe (e.g. Brooks & Mills, 2003; Fialkowski et al., 2003; Dedourge-Geffard et al., 2009; Coulaud et al., 2011). Gammarids are also often more sensitive than *Daphnia magna* Straus, 1820 (Gerhardt, 2011) towards different types of pesticides, such as neurotoxic substances and especially pyrethroids. *Gammarus fossarum* Koch, 1836 (Crustacea; Amphipoda) is a relatively robust and abundantly occurring member of the macrozoobenthos of European springs. It inhabits springs and spring brooks in mountainous regions of central Europe (Janetzky, 1994; Pöckl et al., 2003). *G. fossarum* is a key species (e.g. Dangles et al., 2004) that mainly acts as an efficient shredder, but also feeds on fine particulate organic matter (FPOM) (Moog 1995). It plays a fundamental role in organic matter breakdown in springs and spring brooks and hence in the distribution of coarse particulate organic matter (CPOM) and FPOM (Wagner 1990; Simcic & Brancelj, 2006). *G. fossarum,* a typical inhabitant of running waters rich in oxygen (Lukancic et al., 2009) and low pH, is more sensitive than *Gammarus pulex* (L.) (e.g. Rinderhagen et al., 2000; Alonso et al., 2010) towards contamination of water and low oxygen. For these reasons *G. fossarum* can be deemed a suitable organism for assessing impacts of pollution and it is readily used in ecotoxicological assays (e.g. Westram et al., 2011; Gerhardt, 2011; Maltby et al., 2002).

Laboratory experiments guarantee reproducibility of the results by exactly defining the test conditions. They provide numerous replicates and are suitable for a variety of experiments on different biological levels, for example the population level. Furthermore they are useful tools when assessing contamination effects on certain species. Quite a few studies on laboratory experiments concerning the effects of copper exposure on freshwater species have been published (e.g. Sroda & Cossu-Leguille, 2011; Reichmuth et al., 2010). However, laboratory conditions are, depending on the experimental design, far from natural (e.g. petri dishes, static), usually very standardised and optimised so that species might react differently under natural conditions. Laboratory experiments conducted in flow channels provide a certain degree of reproducibility and control and are much more realistic than other laboratory experiments. Experiments in artificial flow channels are more suitable than other laboratory set-ups, especially for stream invertebrates. Mesocosms have been used in artificial indoor streams (e.g. Böttger et al., 2013) and in a few

natural streams (e.g. Coulaud et al., 2011). Furthermore, flow-through microcosms were used to analyse the impact of elevated temperature on the emergence of the mayfly *Baetis bicaudatus* Dodds (Harper & Peckarsky, 2006). However, the best approach to natural conditions is gained with field experiments, which are generally not easy to establish and usually lack reproducibility but are crucial for understanding for example the real effects of pollutants on certain species in the field.

In this study, we conducted feeding tests and the respiratory Electron Transport System (ETS) assay in order to determine the effects of copper contaminated leaf discs on *G. fossarum* both in the laboratory in artificial flow channels and in the field, using test chambers. The feeding activity is a suitable non-lethal endpoint (Pestana et al., 2007) and gives insight into the metabolic activity of the organisms, on the level of the organism. It has been used widely in the last decade in many different experiments (e.g. Bundschuh et al., 2009; Dedourge-Geffard et al., 2009). The ETS assay was conducted to quantify the effects of the copper on the cellular level of the organisms. The ETS is an enzyme system found in the inner mitochondrial membranes of eucaryotes which controls the oxygen consumption (G.-Toth, 1999) and the results reflect the maximum oxygen consumption when all enzymes are functioning optimally (Kenner & Ahmed, 1975).

Our aims were to find out how *G. fossarum* reacts to a copper-contaminated food source and to see if results from the laboratory differed to those from the field.

2. Materials and Methods

2.1. Sampling site

Specimens were collected from one natural rheocrene in the Röserental near Liestal, in Switzerland (see von Fumetti et al., (2007)). They were pipetted with a turkey baster into white trays and counted out for use in the spring and laboratory. Individuals of both sexes were collected and their average wet weight was 11.1 ± 5.2 mg. Organisms were used regardless of their sex, as has been done in other studies (e.g. Cold & Forbes 2004; Bundschuh et al. 2009; Alonso et al. 2010). Selected specimens showed no sign of being parasitised and their movement was normal. Specimens for use in the laboratory were transported in closed plastic boxes with spring water and

leaves from the spring. They were then kept in the transport containers at 10 °C for 46 hours, for technical reasons not 48 hours owing to transport time, for acclimatisation to laboratory conditions before being used in an experiment.

The temperature of the spring water was 10.4 ± 0.1 °C. The pH was 7.1 ± 0.2 and the electrical conductivity of the spring water was 571.2 ± 17.7 µS / cm. The oxygen concentration was 7.3 ± 1.5 mg / L and the saturation was 68.0 ± 13.9 %. They were all measured using portable meters (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany).

The nutrients phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) of the spring water were measured with Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (SPECTRO MS, Spectro Analytical Instruments GmBh, Kleve, Germany). The nitrate concentration of the spring water was 18.0 mg / L, the nitrite < 0.05 mg / L, the ammonia concentration < 0.1mg / L and the phosphate < 0.5 mg / L.

The copper concentration of the natural spring water measured with ICP-OES was found to be smaller than 0.001 mg Cu / L. The spring water has a ionic composition as follows: potassium (Na^+) 2.5 mg / L, calcium (Ca^{2+}) 106.5 mg / L, magnesium (Mg^{2+}) 7.9 mg / L, chloride (Cl^-) 12.7 mg / L and sulphate ($SO4^{2-}$) 33.9 mg / L. The carbonate hardness is 24.7 mg / L. The spring water exhibits a total hardness of 29.9 mg / L.

2.2. Conditioning of the leaf discs

The spring from which the gammarids were obtained is surrounded mainly by beech trees. These leaves form the most important food source for its inhabitants including *G. fossarum*. Therefore, we collected beech leaves *(Fagus sylvatica* L.*)* from the litter layer near the spring after abscission in autumn 2013. The collected leaves were first washed, dried in an oven at 40 °C and then stored as described by Bloor (2010). Leaf discs (diameter 1 cm) were cut out of the collected leaves using a cork borer. Ten leaf discs were always weighed together and then placed together in numbered stainless steel herb infusers (Ø 9 cm). The infusers were submerged into aerated spring water with fine particulate organic matter from the spring for conditioning. They were conditioned at a water temperature of 17 ± 0.5 °C for four weeks.

2.3. Choice of copper concentration for leaf disc exposure

We conducted pre-tests with beech leaf discs using the following nominal copper concentrations (all in mg Cu / L): 0.4, 0.8, 1.6, 3.2 and 6.4, in order to decide to which copper concentration to expose the leaf discs in our experiments. The range of 0.4 to 6.4 mg Cu / L was chosen loosely on previously conducted LC_{50}-tests (see Schmidlin et al., 2014). We started off with a ten times higher concentration than for the LC_{50}-tests and then always doubled the concentrations in order to obtain a concentration range suitable for testing. Copper sulphate penta-hydrate ($CuSO_4*5H_2O$, Merck, Lot no. A921690 717) was used together with spring water to make up all solutions. The results of the pre-tests suggested using a nominal copper concentration of 0.8 mg Cu / L for leaf discs exposure. After four weeks conditioning time the leaf discs were placed into 250 ml beakers and soaked for 96 hours before use in the experiments: those destined for the control in 200 ml pure spring water and those for the experimental groups in 200 ml of the chosen copper spring water solution. This procedure was based on Abel & Bärlocher, who exposed oak leaf discs to cadmium (1988).

2.4. Experimental design: laboratory and spring

The experiments were carried out both in a laboratory and in a natural spring using test chambers. The test chambers were flow-through systems, consisting of a sheer 95 mm long plastic tube with a diameter of 85 mm and on the one end a mesh net (0.5 mm net size) which was held to the tube by a cable binder and silicon. At the other end was a removable lid for addition of leaf discs and gammarids. The lids were soft plastic where the centre parts were replaced by mesh netting stuck on with silicon and sewn on with fishing line, so that water could flow through the test chambers.

In the laboratory the experiments were conducted in two stainless steel flow channels, one held the experimental group and the other the control group. Each unit consisted of the actual flow channel (1 m x 0.4 m x 0.2 m), a tube through which spring water flowed into a rain barrel (60 litres) functioning as a water reservoir, an aquarium pump (EHEIM, compact 1000, Deizisau, Germany) to pump the water into the channel and a cooling unit (Aqua Medic Titan 500, Blessendorf, Germany). The water temperature was kept at 10 °C with an accuracy of ± 0.5 °C. Spring water

from the spring from which the organisms were collected was used so that the presence of a natural microflora was given (Jonsson & Malmquist, 2000). Five test chambers were placed into every channel; two per channel were for feeding tests and the other three for ETS testing. In the control the gammarids were fed non-contaminated leaf discs and in the experimental groups the gammarids were fed copper contaminated leaf discs. The photoperiod was 11 hours light and 13 hours dark.

The field experiments were conducted in a spring with the amount of copper smaller than 0.001 mg Cu / L in the Swiss Jura, from which the gammarids had been taken. In the field the five test chambers with non-contaminated leaf discs were positioned in the upper reach of the spring and the other five below these. The test chambers were attached with cable binders to large stainless steel nails which were hammered into the bed of the spring.

Both in the laboratory and the field six test organisms were placed into each test chamber. Ten conditioned leaf discs were placed into every test chamber as the food source and for shelter. Each experiment lasted seven days and was repeated a total of six times to obtain six replicates. The experiments were conducted from February to April 2014.

Both in the laboratory and field the test chambers 1, 2, 6 and 7 were destined for feeding tests. The other test chambers were reserved for ETS testing. Test chambers 1 to 5 held copper-free leaf discs; test chambers 6 to 10 held copper-contaminated leaf discs.

In the laboratory the organisms were monitored daily for deaths and these were removed from the test chambers. In the field the test chambers were untouched during the duration of the experiments. Few deaths occurred both in the laboratory and field, the maximum mortality rate being 5 %.

2.5. Copper accumulation: in leaf discs and gammarids

The copper content of the leaf discs and gammarids used in our experiments were determined with ICP-OES (SPECTRO MS, Spectro Analytical Instruments GmBh, Kleve, Germany) by extracting the copper from these tissues. For this, the leaf discs and gammarids were dried at 40 °C, weighed and then placed separately in test tubes containing 10 ml of 2M HNO_3. The tubes were placed in a rack in a boiling water bath for extraction for 2 hours. The content of these tubes was then filtered

and the liquid stored in clean plastic tubes shut tightly. Before measuring the copper content, the leaf disc samples were diluted 5 or 10 times with 3 % HNO_3. The gammarid samples were not diluted in this manner. The leaf discs of the laboratory and spring were analysed separately by their location. Since the sample size of the analysed gammarids was so limited they were analysed together, regardless of their location.

2.6. Feeding activity: laboratory and spring

At the end of the feeding experiments all gammarids were preserved in 100 % ethanol, dried at 40 °C and weighed. The leaf discs on which they had fed were also dried at 40 °C and then weighed. Maltby et al. (2002) and many other authors using feeding activity as an endpoint for their experiments speak of feeding rates; considering that gammarids are shredders and hence do not necessarily eat all the leaf material they process, we prefer to use the term feeding activity. The feeding activity was determined for every box, according to Maltby et al. (2002), for the 6 organisms together, as described below:

$$FA = ((Li \times \text{Control factor}) - Lf) / (\text{weight} \times \text{time})$$

where FA is the feeding activity, Li is the initial dry weight (mg) of the leaves, Control factor is the loss of weight of leaves during 7 days when no feeding takes place (experimentally tested), Lf is the final dry weight (mg) of the leaves, weight is the dry weight of gammarids (mg) and time is the duration of experiment (days).The dry weight of six gammarids together, as used in the feeding tests, was on average 14.5 ± 3.5 mg.

2.7. ETS activity: laboratory and spring

The ETS activity of the test organisms was determined according to the method originally developed by Packard (1971) and improved by G.-Toth (1999). The ETS activity was always measured at the end of every experiment. All six specimens from one test chamber were removed and placed together in a numbered plastic tube which was immediately put into a container with

liquid nitrogen, LN2 voyageur 5 (Carbagas, Gümligen, Switzerland). This was done both in the laboratory and field. The tubes were removed from the liquid nitrogen container after 1 hour and stored in a fridge at 4 °C for approximately 20 hours until being analysed. Liquid nitrogen has been used before in the ETS assay for fixation of *Daphnia magna* (De Coen, 2003) and of tissue of crayfish (Simcic et al., 2014).

In total 18 test organisms per treatment giving a total of 36 gammarids per experiment, both in the laboratory and field, giving a total of 72 gammarids, were analysed.

To conduct the ETS assay nine gammarids were removed from the plastic tubes, one by one, placed on a tissue and gently patted dry. They were then weighed singly on a microbalance (XP6, METTLER TOLEDO, Greifensee, Switzerland), and placed on a numbered aluminium sheet. The gammarids were then homogenised singly in the homogenisation tube, using 4ml of ice-cold homogenising buffer solution [0.1 M sodium phosphate buffer pH 8.4, 75 µM $MgSO_4$, 0.15 % (w/v) polyvinyl pyrrolidone, 0.2 % (v/v) Triton-X-100]. The homogenate was poured into a centrifuge tube and sonicated with an ultrasonic homogeniser (BandelinSonopuls HD2070, Berlin, Germany) for 20 seconds and stored in an ice solution. The homogenate was then centrifuged (Sigma 2-16 PK, Osterode am Harz, Germany) at 0 °C for 4 minutes at 10000 r.p.m., according to Simcic & Brancelj (2004). The supernatant (in triplicate) was incubated with 1.5 mL substrate solution [0.1 M sodium phosphate buffer pH 8.4, 1.7 mM NADH, 0.25 mM NADPH, 0.2 % (v/v) Triton-X-100] and 0.5 mL reagent solution [2.5 mM 2-p-iodo-phenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride] for 40 minutes at 10 °C. Stopping solution [formaldehyde (conc.):H_3PO_4 (conc.) = 1:1], 0.5 mL, was added immediately after incubation and the formazan production determined spectrophotometrically with the spectroquant® Pharo 300 (Merck, Darmstadt, Germany) by measuring the absorbance of the sample at 490 nm against the blank.

In order to obtain a conversion to equivalent oxygen the ETS activity was calculated according to Kenner & Ahmed (1975) as follows:

$$\text{ETS activity } (\mu L\ O_2 / mg \times h) = ((Abs^{490nm} \times Vr \times Vh \times 60) / (Va \times Gw \times t \times 1.42)$$

where Abs^{490nm} is the absorption of the sample, Vr is the final volume of the reaction mixture (3 mL), Vh is the volume of the original homogenate (4 mL), Va is the volume of the aliquot of the homogenate (0.5 mL), Gw is the gammarid weight (mg), t is the incubation time (minutes) and 1.42 is the factor for conversion to volume oxygen. The weight of a single gammarid used in the ETS experiments was on average 10.7 ± 5.2 mg.

2.8. Data analysis

Standard calculations were done with Microsoft Office Excel 2007. The analyses of variance (ANOVAs) were calculated using the Statistical Package for Social Sciences (SPSS) version 22 for Windows (SPSS Inc, Chicago, IL, U.S.A.). Two-way and one-way ANOVAS were conducted to test the copper influence (contaminated leaf discs) and the influence of location (laboratory or spring) on the feeding and ETS activities of the test organisms. Variance homogeneity was tested with the Levene-Test and the data variance was found to be homogeneous. The α-level was set at 0.05.

3. Results

3.1. Copper accumulation: in leaf discs and gammarids

The average copper content in the leaf discs not exposed to copper was 0.09 ± 0.02 mg Cu / g leaf disc. The average copper content in the leaf discs exposed to copper was 0.7 ± 0.9 mg Cu / g leaf disc. The lowest value was measured in leaf discs not exposed to copper in the laboratory and the highest in leaf discs exposed to copper in the laboratory. The mean copper content in the leaf discs not exposed to copper were all lower than the copper content in the leaf discs exposed to copper.

A one-way ANOVA comparing the different treatments (copper-free & copper-exposed) revealed a significant effect of the copper on the copper content of the leaf discs in the laboratory, $F_{1, 25}= 6.73$, $p = 0.018$ and in the spring, $F_{1, 25} = 10.81$, $p = 0.003$ in that those leaf discs exposed to copper had significantly more copper than those not exposed (Fig. 1). A comparison of the locations (laboratory and spring) under no copper influence revealed no significant effect on the copper content of the leaf discs, $F_{1, 25} = 0.18$, $p = 0.674$. A comparison of the locations under copper

influence also revealed no significant effect on the copper content of the leaf discs, $F_{1, 25} = 1.70$, $p = 0.204$ (Fig. 1).

The mean copper content in the gammarids ranged from 0.05 to 0.11 mg Cu / g of gammarid tissue. The lowest value was measured in a gammarid not exposed and the highest in a gammarid exposed to copper. A one-way ANOVA comparing the different treatments revealed no significant effect on the copper content of the gammarids, $F_{1, 11} = 3.00$, $p = 0.111$ (Fig. 1).

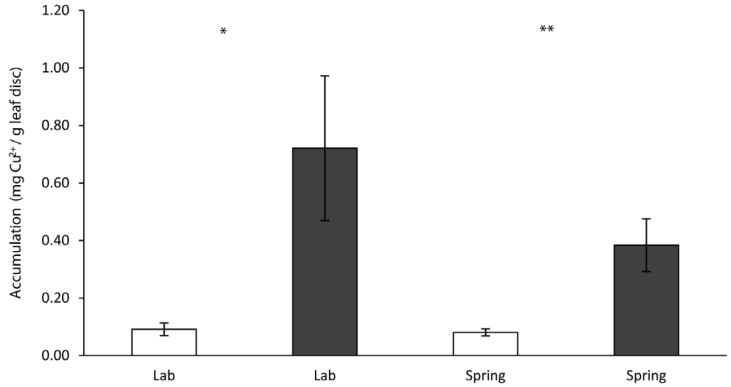

Fig. 1 Mean copper ion content of the leaf discs both in the laboratory and in the spring. Standard errors are represented by the error bars attached to each column. White: copper-free, Dark grey: copper-exposed. Note: treatment ** < 0.004, * < 0.05

3.2. Feeding activity: laboratory and spring

The average feeding activity was 0.12 ± 0.06 mg / mg × d. Both the lowest and highest value was measured in a test chamber exposed to copper in the spring.

A two-way ANOVA over all the feeding activity data showed that the treatment (copper-free and copper-exposed) did not have a significant effect on the feeding activity of the gammarids $F_{1, 44} = 0.106$, $p = 0.747$ but the location (laboratory and spring) had a highly significant effect on the feeding activity of the gammarids $F_{1, 44} = 12.724$, $p = 0.001$. The interaction of treatment and location was found to be insignificant, $F_{1, 44} = 0.732$, $p = 0.397$.

A one-way ANOVA comparing the different treatments revealed no significant effect on the feeding activity in the laboratory, $F_{0, 22} = 0.17$, $p = 0.680$ or in the spring, $F_{1, 22} = 0.58$, $p = 0.451$.

A comparison of the locations under no copper exposure revealed a significant effect on the feeding activity, $F_{1, 22} = 4.16$, $p = 0.053$, in that the feeding activity of the gammarids in the laboratory was higher than of those in the spring (Fig. 2). A comparison of the locations with copper exposure revealed a significant effect on the feeding activity, $F_{1, 22} = 8.75$, $p = 0.007$, in that the feeding activity of the gammarids in the laboratory was higher than of those in the spring (Fig. 2).

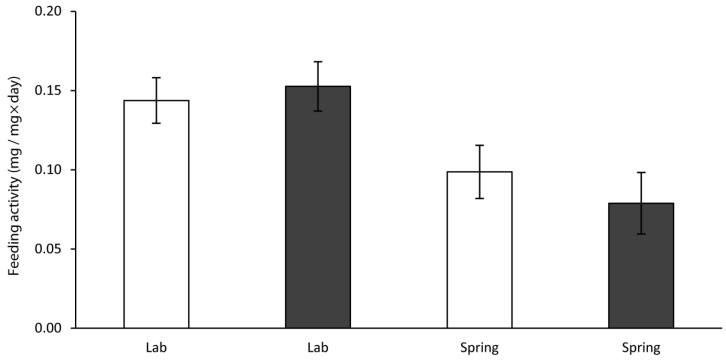

Fig. 2 Calculated mean feeding activity of *G. fossarum*in the laboratory and in the spring. The data was obtained between February and May 2014 and is pooled. Standard errors are represented by the error bars attached to each column. White: copper-free, Dark grey: copper-exposed.

3.3. ETS activity: laboratory and spring

The average ETS activity was 0.6 ± 0.3 µL O_2 / mg × h. The lowest activity was measured in a gammarid exposed to copper in the spring and the highest in a gammarid exposed to copper in the laboratory. The mean ETS activities of the gammarids not exposed to copper were all higher than the ETS activities of the gammarids exposed to copper.

A two-way ANOVA over all the ETS data showed that both the treatment (copper-free and copper-exposed) $F_{1, 418} = 32.914$, $p < 0.001$ and the location (laboratory and spring) had a highly significant effect on the ETS activity of the gammarids $F_{1, 418} = 32.914$, $p < 0.001$ and $F_{1, 418} = 38.014$, $p < 0.001$, respectively. The interaction of treatment and location was also found to be significant, $F_{1, 418} = 5.464$, $p = 0.020$.

A one-way ANOVA comparing the different treatments revealed a highly significant effect on the ETS activity in the laboratory, $F_{1, 206}$= 29.49, p < 0.001 and in the spring, $F_{1, 212}$ = 6.42, p = 0.012 in that the copper reduced the ETS activity of the gammarids (Fig. 3). A comparison of the locations with no copper exposure revealed a highly significant effect on the ETS activity of the gammarids, $F_{1, 208}$= 36.56, p < 0.001, in that the ETS activity of the gammarids in the laboratory was higher than of those in the spring (Fig. 3). A comparison of the locations with copper exposure revealed a significant effect on the ETS activity, $F_{1, 220}$= 7.29, p = 0.008, in that the ETS activity of the gammarids in the laboratory was higher than of those in the spring (Fig. 3).

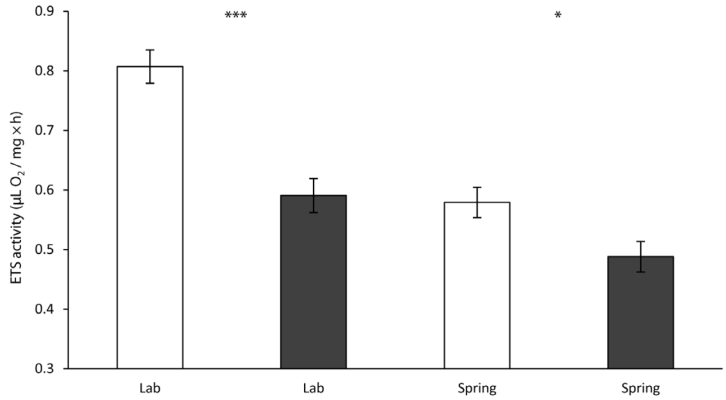

Fig. 3 Mean ETS activities of *G. fossarum* both in the laboratrory and spring. The data was obtained between February and May 2014 and is pooled. Standard errors are represented by the error bars attached to each column. White: copper-free, Dark grey: copper-exposed. Note: treatment *** < 0.001, * < 0.05

4. Discussion

4.1. Copper accumulation

Heavy metals affect the metabolic activity of organisms, their behaviour and their distribution in the ecosystems (e.g. Lemus & Chung, 1999). It is known that many freshwater species, such as the highly endangered freshwater pearl mussels (Nagel, 1990) and amphipods, accumulate metals in their systems by absorption from the surrounding water and sediment or through ingestion of food (e.g. Abel & Bärlocher, 1988; Weeks, 1992). In our experiments it was shown that copper was absorbed by leaf discs exposed to a copper solution for 96 hours. Obviously adsorption of the

copper to the leaf discs took place, as has been previously described (Tattersfield, 1993). The location (laboratory versus spring) did not have an effect on the concentration of copper in the leaf discs. It can be assumed that an eluviation of the copper did not occur, and the adsorption was stable. Our experiment is therefore a realistic approach for assessing the impact of contaminated food on macroinvertebrates.

The copper concentration of the gammarids fed with contaminated and non-contaminated leaf discs did not differ significantly; there was no net accumulation of copper, this means that uptake and excretion were balanced. Reasons for the insignificance may lay in the fact that sample sizes were too small for statistical testing. It has recently been concluded that *G. pulex* is a suitable biomonitor to quantify the bioavailabe fraction of copper in freshwaters (Lebrun et al., 2012). This also seems to be true for *G. fossarum*. Copper ions are essential for oxygen transport in haemocyanin, but if the up-take is in excess, then accumulation can occur (Reichmuth et al., 2010). Long-term accumulation has been postulated to be caused by contaminated food sources (Schwörbel & Brendelberger, 2005), which makes investigations into contaminated potential food sources even more important when considering pollution of the environment.

4.2. Feeding and ETS activity

In the laboratory the feeding activity of the gammarids fed on copper contaminated leaf discs was slightly higher than in the control groups. This is surprising, as copper acts as a fungicide and therefore the leaf discs were probably not as rich in living fungi as those from the control groups. It has been shown that *G. fossarum* preferred leaf discs conditioned in the presence of an antibiotic mixture over those in the control (Bundschuh et al., 2009). In a further study *G. fossarum* avoided leaf discs conditioned in the presence of the fungicide tebuconazole (Bundschuh et al., 2011). In the spring, the feeding activity of the gammarids in the experimental groups was slightly lower than those in the control group. This result was expected. However, since the differences were not significant, the results in the laboratory which differ slightly compared to the spring should not be given too much attention. The hypothesis that copper would lower the metabolic activity of *G. fossarum* on the level of the organism has to be rejected when the feeding activity is used as a

measure. Obviously the contaminated leaf discs were still palatable for the gammarids and so the feeding activity does not reflect the contamination. Also, it should be pointed out that only a limited amount of replicates were used when determining the feeding activity and so the results should not be overrated.

Our ETS results show that a significant change on the cellular level of the organisms was caused by exposure to a copper contaminated food source, as had been hypothesised. *G. fossarum* exposed to copper contaminated leaf discs showed significant decreases in ETS activity when compared to *G. fossarum* fed with non-contaminated leaf discs, in both the laboratory and the spring. Copper ions are regulated in all gammarid species (Taylor & Anstiss, 1999) and these have detoxification mechanisms to counteract toxicity by metal ions (Geffard et al., 2010). However, if these detoxification mechanisms are unable to regulate the excess of internalized metal, the excess leads to physiological disturbances (Lebrun et al., 2012).This has been shown in this study with the ETS assay. Besse et al. (2013) concluded that gammarids can be regarded as poor indicators for copper (Besse et al., 2013). Like Lebrun et al. (2012) we have also been able to demonstrate in this study that the ETS activity is affected by this metal.

In the laboratory the difference of the ETS activities between the control and experimental groups was highly significant. A reason for this can be found in the results of the feeding activity: the gammarids consumed most leaf litter in the test chambers containing copper contaminated leaf discs which is directly reflected in the ETS activity. Another explanation for this observation could be that ingested copper was excreted back into the water and since the water was circulated in the laboratory, the water immediately contained more copper than the water in the spring – the water in the spring was constantly renewed, and so any excreted copper would have been flushed away.

4.3. Laboratory versus field experiment

We hypothesised that the feeding and ETS activities of *G. fossarum* would generally be lower in the laboratory than in the spring but the opposite was found to be true: the feeding and also ETS activity of the organisms in the spring were significantly lower than those in the laboratory, regardless of the treatment.

We assumed that the values obtained in laboratory experiments would be lower than in nature because of the artificial laboratory conditions. This is however not necessarily the case, as our results demonstrate. If the conditions in the laboratory are optimal, the exposed organisms are in fact exposed to more favourable conditions than in nature. In nature, organisms are often exposed to suboptimal conditions and these affect their behaviour and response towards contaminants and other environmental changes. In the field, the gammarids in the test chambers were exposed to rain, wind and other disturbances, which may have caused differences in the chemical composition of the spring water. It is generally agreed that organisms living under environmental conditions that are near to their tolerance limits are often less resistant to additional stressors, such as exposure to pollutants (Heugens, 2003). In previous experiments with fluctuating temperatures, we concluded that a water temperature of 12 to 14 °C was probably more optimal for *G. fossarum* (Schmidlin et al., 2014). The gammarids in the spring are exposed to 10 °C throughout the year and although the gammarids in the laboratory were also exposed to 10 °C, they were not subjected to additional stressors when compared to those in the spring. Our results suggest that all these disturbance factors in the spring were responsible for the lower feeding and ETS activity. Another explanation could be that the feeding activity in the spring was influenced by the gammarids feeding on incoming particles and small organisms, which we were not able to quantify. It can also be argued that elevated feeding activity in the laboratory could be a response to stress and so the metabolic activity of the gammarids was higher.

4.4. Implications for spring ecology

The contamination of our environment has taken on new levels since the frequent and wide spread use of various substances such as pharmaceuticals and pesticides. Metals are being introduced into the environment for example through mining, paint and via fungicides. Copper is introduced into the environment mainly through non-point sources such as road and roof run-off. Laboratory studies testing for a certain substance and its effects are important; however, the environment is exposed to a variety of mixtures of contaminants and is exposed to many environmental disturbances. This shows that it is very important to make use of bioindicators directly in the field

and that field experiments are vital. *G. pulex* is widely used as a bioindicator (Gerhardt, 2011), as well as *G. fossarum*, although less frequently. *G. fossarum* has a narrower distribution than *G. pulex* because it is more sensitive to environmental variables and towards pollutants (Alonso et al., 2010) and is the more vulnerable of the two competitors. This species was chosen in this experiment as a representative of the European spring fauna, while it is not cold-stenothermal, it is still sensitive to environmental contaminants. Other spring inhabitants such as Trichoptera and Plecoptera larvae can be considered less resilient towards contamination, for example by copper, than *G. fossarum*. These species are adapted to the relatively stable environmental conditions (e.g. Danks & Williams, 1991; Ferrington, 1995) they find in these locally very restricted ecotones (Webb et al., 1998). Springs can be strongly influenced by disturbances such as drought or heavy rainfall (von Fumetti & Nagel, 2012) and the occurring spring species are especially sensitive towards these influences. Although springs are known for their clean water, pollution can occur through entry of contaminated leaf matter and run-off of contaminants directly into the water or via the groundwater or via soil erosion. Depending on the characteristics of the contaminants these can accumulate in the leaf litter in the spring via adsorption as is shown in our experiments and are thus not flushed away, but stay in the systems for a long period of time. Ingestion of the contaminated leaf litter through invertebrates promotes bioaccumulation. Our study clearly demonstrates the impacts contaminated leaf litter, at a sublethal level, can have on *G. fossarum* in springs by significantly lowering their ETS activity. A similar or greater impact on more sensitive spring species can be expected. *G. fossarum*'s function as a bioindicator should be used as such.

4.5. Benefits of field experiments

Many laboratory experiments have been conducted with gammarids in recent years, with some using artificial indoor streams (e.g. Böttger et al., 2013; Prato et al., 2013; Berghahn et al., 2012; Jubeaux et al., 2012; Bundschuh et al., 2011). In comparison, fewer field studies have been conducted using this taxon (but see e.g. Besse et al., 2013; Gerhardt et al., 2012; Coulaud et al., 2011; Dangles & Guérold, 2000). Generally speaking, laboratory investigations cannot fully simulate or form a substitute for field studies under actual environmental conditions (Nagel, 1995).

Our results demonstrate how important field experiments are also for ecotoxicology, since extrapolations of results from the laboratory to the field are not always accurate. In our study we were however able to demonstrate that extrapolations are possible, as was also shown by Selck et al. (2002). The ultimate aim of ecotoxicology is to determine and predict the effects of contaminants in real-world systems, optimally at large spatial scales (e.g. Newman and Unger, 2003). It is still difficult to predict the effects of toxicants on real-world ecosystems at large spatial scales, as the capacity for these remains limited (Beketov & Liess, 2012). In a quantitative analysis of data, where uncertainty factors for laboratory to field extrapolations were calculated, it was ascertained that the toxicity under laboratory and relevant field conditions differed by factors of 2.6 to 130 depending on the examined effect parameters (Heugens, 2003). It was shown by Versteeg et al. (1999) that sufficiently large data sets from laboratory-generated chronic tests can be used to define concentrations protective of model ecosystems. The combination of laboratory and field tests is essential for environmental risk assessments. We made a first step in this direction by conducting a field experiment with a number of replicates and without harming the entire spring fauna, while still simulating a realistic scenario. Such experiments should be promoted in future freshwater studies.

5. Conclusions

The results of this study show that the exposure to copper in excess has the potential of being stressful to *G. fossarum*. Copper did not affect the feeding activity but significantly reduced the ETS activity of *G. fossarum*. The ETS assay has proved to be more sensitive than the feeding tests in detecting the effects of sub-lethal copper concentrations on the metabolic activity of *G. fossarum*. We therefore propose the use of the ETS assay in addition to the well-established feeding tests for more detailed results. Furthermore we demonstrated the importance of conducting experiments both in a laboratory and directly in the field, as the results cannot necessarily be extrapolated. The feeding activity differed, although not in a way that meaningfully affected toxicity. However, it could be shown that the effect of copper on the ETS activity of the exposed gammarids was comparable between the laboratory and field studies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Abel, T., Bärlocher, F., 1988. Uptake of cadmium by *Gammarus fossarum* (Amphipoda) From Food and Water. Journal of Applied Ecology 25:1:223-231.

Ahmed, S.I., Kenner, R.A., King, F.D., 1976. Preservation of enzymic activity in marine plankton by low-temperature freezing. Marine Chemistry 4:133-139.

Alonso, A., De Lange, H.J., Peeters, E.T.H.M., 2010. Contrasting sensitivities to toxicants of the freshwater amphipods *Gammarus pulex* and *G. fossarum*. Ecotoxicology 19: 133-140.

Beketov, M.A., Liess, M., 2012. Ecotoxicology and macroecology – Time for integration. Environmental Pollution 162:247-254.

Berghahn, R., Mohr, S., Hübner, V., Schmiediche, R., Schmiedling, I., Svetich-Will, E., Schmidt, R., 2012. Effects of repeated insecticide pulses on macroinvertebrate drift in indoor stream mesocosms. Aquatic Toxicology 122-123:56-66.

Besse, J.P., Coquery, M., Lopes, C., Chaumot, A., Budzinski, H., Labadie, P., Geffard, O., 2013. Caged *Gammarus fossarum* (Crustacea) as a robust tool for the characterization of bioavailable contamination levels in continental waters: Towards the determination of threshold values. Water Research 47: 650-660.

Bloor, M., 2009. Aquatic Pollution. Case Study of Landfill Leachate Toxicity and Remediation. VDM Verlag.

Bloor, M.C., 2010. Animal standardisation for mixed species ecotoxicological studies: Establishing a laboratory breeding programme for *Gammarus pulex* and *Asellusaquaticus*. Zoologica baetica 21: 179-190.

Böttger, R., Feibicke, M., Schaller, J., Dudel, G., 2013. Effects of low-dosed imidacloprid pulses on the functional role of the caged amphipod *Gammarus roeseli* in stream mesocosms. Exotoxicology and Environmental Safety 93:93-100.

Brooks, S.J., Mills, C.L., 2003. The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comparative Biochemistry and Physiology Part A 135: 527-537.

Bundschuh, M., Hahn, T., Gessner, M.O., Schulz, R., 2009. Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 28: 197-203.

Bundschuh, M., Zubrod, J.P., Kosol, S., Maltby, L., Stang, C., Duetser, L., Schulz, R., 2011. Fungal composition on leaves explains pollutant-mediated indirect effects on amphipod feeding. Aquatic Toxicology 104: 32-37.

Cold, A., Forbes, V.E., 2004. Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. Aquatic Toxicology 67: 287-299.

Coulaud, R., Geffard, O., Xuereb, B., Lacaze, E., Quéau, H., Garric, J., Charles, S., Chaumot, A., 2011. *In situ* feeding assay with *Gammarus fossarum* (Crustacea): Modelling the influence of confounding factors to improve water quality biomonitroing. Water Research 45: 6417-6429.

Dangles, O.J., Guérold, F.A., 2000. Feeding activity of *Gammarus fossarum* (Crustacea: Amphipoda) in acidic and low mineralized streams. Verhandlungen des Internationalen Verein Limnologie 27: 1-4.

Dangles, O., Gessner, M.O., Guérold, F., Chauvet, E., 2004. Impacts of stream acidification on litter breakdown: implications for assessing ecosystem functioning. Journal of Applied Ecology 41: 365-378.

Danks, H.V., Williams, D.D., 1991. Arthropods of springs, with particular reference to Canada: Synthesis and needs for research. In Williams, D. D. & H. V. Danks (eds), Arthropods of springs, with particular reference to Canada. Memoirs of the Entomological Society of Canada 155: 203–217.

Debelius, B., Forja, J.M., DelValls, A., Lubian, L., 2009. Toxicity and bioaccumulation of copper and lead in five marine microalgae. Ecotoxicology and Environmental Safety 72: 1503-1513.

Dedourge-Geffard, O., Palais, F., Biagianti-Risbourg, Geffard, O., Geffard, A., 2009. Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: An *in situ* experiment. Chemosphere 77: 1569-1576.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of the toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environmental Toxicology and Chemistry 22: 1632-1641.

De Martinez Gaspar Martins, M., Barcarolli, I.F., De Menezes, E.J., Giacimin, M.M., Wood, C.M., Bianchini, A., 2011. Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectessapidus* acclimated to different salinities: *In vivo* and *in vitro* studies. Aquatic toxicology 101: 88-99.

De Oliveira-Filho, E.C., Lopes, R.M., Paumgartten, F.J.R., 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56: 369-374.

Felten, V., Charmantier, G., Mons, R., Geffard, A., Rousselle, P., Coquery, M., Garric, J., Geffard, O., 2008. Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquatic Toxicology 86: 413-425.

Ferrington, L.C., 1995. Biodiversity of aquatic insects and other invertebrates in springs: Introduction. Journal of the Kansas Entomological Society 68: 1-3.

Fialkowski, W., Fialkowski, E., Smith, B.D., Rainbow, P.S., 2003. Biomonitoring Survey of Trace Metal Pollution in Streams of a Catchment Draining a Zinc and a Lead Mining Area of Upper Silesia, Poland Using the Amphipod *Gammarus fossarum*. International Review of Hydrobiology 88:2:187-200.

Fleituch, T., 2003. Structure and Functional Organization of Benthic Invertebrates in a Regulated Stream. International Review of Hydrobiology 88:3- 4:332-344.

Geffard, A., Sartelet, H., Garric, J., Biagianti-Risbourg, S., Delahaut, L., Geffard, O., 2010. Subcellular compartmentalization of cadmium, nickel, and lead in *Gammarus fossarum*: comparison of methods. Chemosphere 78:822-829.

Gerhardt, A., 2011. GamTox: A Low-Cost MultimetricEcotoxicitytet with *Gammarus* spp. for *In* and *Ex Situ* Application. International Journal of Zoology, 1-7.

Gerhardt, A., Koster, M., Lang, F., Leib, V., 2012. Active *in Situ*Biomonitroing of Pesticide Pulses Using *Gammarus spp*. in Small Tributaries of Lake Constance. Journal of Environmental Protection 3:573-583.

G.-Toth, L., 1999. Aktivität des Elektronentransportsystems. In: Biologische Gewässeruntersuchung. Methoden der Biologischen Wasseruntersuchung 2 (Eds W. von Tümpling & G. Friedrich), pp. 465–473. Gustav Fischer Verl., Jena, Stuttgart, Lübeck, Ulm.

Harper**,** M.P., Peckarsky, B.L., 2006. Emergence cues of a mayfly in a high-altitude stream ecosystem: potential response to climate change. Ecological Applications 16:2: 612-621

Hester, E.T., Doyle, M.W., 2011. Human impacts to river temperature and their effects on biological processes: a quantitative syntheses. Journal of American Water Resources Association 47:3:571-587.

Heugens, E.H.W., 2003. Predicting Effects of Multiple Stressors on Aquatic Biota. ProefschriftUniversiteit van Amsterdam.

IPCC 2007. Climate Change, 2007. Synthesis Report. Contribution of working Groups 1, 2 and 3 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. (Eds. Pachauri, R. K. &Reisinger, A.). IPCC, Geneva, Switzerland.

Janetzky, W., 1994. Distribution of the genus *Gammarus* (Amphipoda: Gammaridae) in the River Hunte and its tributaries (Lower Saxony, northern Germany). Hydrobiologia 294: 23-34

Jonsson, M., Malmquist, B., 2000. Ecosystem process rate increases with animal species richness: evidence from leaf-eating, aquatic insects. OIKOS 89: 519-523.

Jubeaux, G., Simon, R., Salvador, A., Lopes, C., Lacaze, E., Quéau, H., Chaumot, A., Geffard, O., 2012. Vitellogenin-like protein measurement in caged *Gammarus fossarum* males as a biomarker of endocrine disruptor exposure: Inconclusive experience. Aquatic Toxicology 122-123: 9-18.

Kenner, R.A., Ahmed, S.I., 1975. Measurements of electron transport activities in marine phytoplancton. Marine Biology 33:119-127.

Ladewig, V., Jungmann, D., Köhler, H.-R., Schirling, M., Triebskorn, R., Nagel, R., 2006. Population Structure and Dynamics of *Gammarus fossarum* (Amphipoda) Upstream and Downstream from Effluents of Sewage Treatment Plants. Archives of Environmental Contamination and Toxicology 50: 370-383.

Lebrun, J.D., Perret, M., Geffard, A., Gourlay-Francé, C., 2012. Modelling copper bioaccumulation in *Gammarus pulex* and alterations of digestive metabolism. Ecotoxicology 21: 20022-2030.

Lemus, M.J., Chung, K.S., 1999. Effect of Temperature on Copper Toxicity, Accumulation and Purification in Tropical Fish Juveniles *PeteniaKraussii* (Pisces: Cichlidae). Caribbean Journal of Science 35: 64-69.

Lindegaard, C., Brodersen, K.B., Wiberg-Larsen, P., Skriver, J., 1998. Multivariate analyses of macrofaunal communities in Danish springs and springbrooks. Studies in Crenobiology. The Biology of Springs and Springbrooks (Ed. L.Botosaneanu), 201-219. Backhuys Publishers, Leiden, the Netherlands.

Lukancic, S., Zibrat, U., Mezek, T., Jerebic, A., Simcic, T., Brancelj, A., 2009. Effects of exposing two non-target crustacean species, *Asellusaquaticus* L., and *Gammarus fossarum* Koch, to Atrazine and Imidacloprid. Bulletin of Environmental Contamination and Toxicology 84: 85-90.

Maltby, L., Clayton, S.A., Wood, R.M., McLoughlin, N., 2002. Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. Environmental Toxicology and Chemistry 21: 361-368.

Moog, O., 1995. Fauna Aquatica Austriaca. Wasserwirtschaftskataster, Bundesministerium für Land- und Forstwirtschaft, Wien.

Nagel, P., 1990. Toxische Metalle als mögliche Ursachen für die Bestandsgefährdung einer Flussperlmuschelpopulation (*Margaritifera margaritifera*). Decheniana 143:412-425.

Nagel, P., 1995. Environmental monitoring handbook for tsetse control operations. MargrafVerlag, Weikersheim, Germany.

Naylor, C., Maltby, L., Calow, P., 1990. Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. Hydrobiologia 188/189: 517-523.

Newman, M.C., Unger, M.A., 2003. Fundamentals of Ecotoxicology. Lewis Publishers.

Packard, T.T., 1971. The measurement of respiratory electron transport system activity in marine phytoplankton. Journal of Marine Research 29: 235-244.

Pantani, C., Pannunzio, G., De Christofaro, M., Novelli, A.A., Salvatori, M., 1997. Comparative Acute Toxicity of Some Pesticides, Metals, and Surfactants to *Gammarus italicus*Goedm. and*Echinogammarustibaldii* Pink. and Stock (Crustacea: Amphipoda). Bulletin of Environmental Contamination & Toxicology 59: 963-967.

Pestana, J.L.T., Re, A., Nogueira, A.J.A., Soares, A.M.V.M., 2007. Effects of Cadmium and Zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyradesmarestii* (Decapoda) and *Echinogammarusmeridionalis* (Amphipoda). Chemosphere 68: 1556-1562.

Pinho, G.L.L., Bianchini, A., Rouleau, C., 2011. Whole-body autoradiography: An efficient technique to study copper accumulation and body distribution in small organisms. Chemosphere 85: 1-6.

Pöckl, M., Webb, B.W., Sutcliffe, D.W., 2003. Life history and reproductive capacity of *Gammarus fossarum* and *G. roeseli* (Crustacea: Amphipoda) under naturally fluctuating water temperatures: a simulation study. Freshwater Biology 48: 53-66.

Prato, E., Parlapiano, I., Biandolino, F., 2013. Sublethal effects of copper on some biological traits of the amphipod *Gammarus aequicauda* reared under laboratory conditions. Chemosphere, 93:6: 1015-1022.

Reichmuth, J.M., Weis, P., Weis, J.S., 2010. Bioaccumulation and depuration of metals in blue crabs (*Callinectessapidus*Rathbun) from contaminated and clean estuary. Environmental Pollution 158: 361-368.

Rinderhagen, M., Ritterhof, J., Zauke, G., 2000. Crustaceans as Bioindicators. From: Biomonitoring of Polluted Water - Reviews on Actual Topics (A. Gerhardt, ed.), Trans Tech Publications - Scitech Publications, Environmental Research Forum 9: 161-194.

Ruyters, S., Salaets, P., Oorts, K., Smolders, E., 2013. Copper toxicity in soils under established vineyards in Europe: A survey. Science of Total Environment 443: 470-477.

Schmidli, J., Schmutz, C., Frei, C., Wanner, H. &Schär, C., 2002. Mesoscale precipitation variability in the region of the European Alps during the 20th century. International Journal of Climatology, 22: 1049-1074.

Schmidli, J., Frei, C., 2005. Trends of heavy precipitation and wet and dry spells in Switzerland during the 20th century. International Journal for Climatology 25: 753 – 771.

Schmidlin, L., von Fumetti, S., Nagel, P., 2014. Effects of increased temperatures on *Gammarus fossarum* under the influence of copper sulphate. Ecotoxicology, DOI 10.1007/s10646-014-1392-6

Schönborn, W., Risse-Buhl, U., 2013. Lehrbuch der Limnologie. 2. Vollständig überarbeitet Auflage. Schweizerbart, Stuttgart.

Schwörbel, J., Brendelberger, H., 2005. Einführung in die Limnologie , 9. Auflage.Spektrum Akademischer Verlag.

Selck, H., Riemann, B., Christoffersen, K., Forbes, V.E., Gustavson, K., Hansen, B.W., Jacobsen, J.A., Kusk, O.K.., Petersen, S., 2002. Comparing sensitivity of ecotoxicological effect endpoints between laboratory and field. Ecotoxicology and Environmental Safety 52: 97-112.

Simcic, T., Brancelj, A., 2004. Respiratory electron transport system (ETS) activity as an estimator of the thermal tolerance of two *Daphnia* hybrids. Journal of Plankton research 26: 525-534.

Simcic, T., Brancelj, A., 2006. Effects of pH on electron transport system (ETS) activity and oxygen consumption in *Gammarus fossarum*, *Asellusaquaticus* and *Niphargussphagnicolus*. Freshwater Biology 51: 686-694.

Simcic, T., F. Pajk, M. Jaklic, A. Brancelj& A. Vrezec, 2014. The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. Journal of Thermal Biology, 41:21-30

Sridhar, K.R., Krauss, G., Bärlocher, F., Raviraja, N.S., Wennrich, R., Baumbach, R., Krauss, G.-J., 2001. Decomposition of alder leaves in two heavy metal-polluted streams in central Germany. Aquatic Microbiolal Ecology 26:73-80

Sroda, S., Cossu-Leguille, C., 2011. Effects of sublethal copper exposure on two gammarid species: which is the best competitor? Ecotoxicology 20: 264-273.

Taylor, H.H., Anstiss, J.M., 1999. Copper and haemocyanin dynamics in aquatic invertebrates. Marine and Freshwater Research 50:8: 907-931.

Tattersfield, L.J., 1993. Effects of copper on the energy budget of a stream detritivore: validation and ecological relevance. Thesis submitted to the University of Sheffield for the degree of doctor of philosophy in the department of animal and plant sciences.

Versteeg, D. J., Belanger, S.E., Carr, G.J., 1999. Understanding single-species and model ecosystem sensitivity: data-based comparison. Environmental Toxicology and Chemistry 18:6: 1329-1346.

Von Fumetti, S., Nagel, P., 2012. Discharge variability and its effect on faunistic assemblages in springs. Freshwater Science 31: 647-656.

Wagner, R., 1990. Influence of Temperature, Photoperiod and Nutrition on Growth and Consumption of Chaetopteryx Villosa (Trichoptera). Holacrtic Ecology 13: 3: 247-254

Webb, D.W., Wetzel, M .J., Reed, P. C., Philippe, L.R. & Young, T.C., 1998. The macroinvertebrate biodiversity, water quality, and hydrogeology of ten karst springs in the Salem Plateau of Illinois. In: Botosaneanu, L. (ed.), Studies in crenobiology: the biology of springs and springbrooks. Backhuys Publishers, Leiden: 39-48.

Weeks, J.M., 1992. The use of the terrestrial amphipod *Arcitalitrusdorreini* (Crustacea; Amphipoda; Talitridae) as a potential biomonitor of ambient zinc and copper availabilities in leaf-litter. Chemosphere 24:10:1505-1522.

Westram, A.M., Jokela, J., Baumgartner, C., Keller, I., 2011. Spatial Distribution of Cryptic Species Diversity in European Freshwater Amphipods (*Gammarus fossarum*) as Revealed by Pyrosequencing. PLoS ONE 6:8: e23879

Woodward, G., Friberg, N., Hildrew, A.G., 2009. The need for scientific rigour in biomonitoring and conservation of fresh waters. Freshwater ecosystems: biodiversity, management and conservation. Hauppage, NY: Nova.

Zollhöfer, J.M., 1997. Quellen - die unbekannten Biotope im Schweizer Jura und Mittelland. Erfassen -Bewerten - Schützen. Bristol Stiftung, Zürich.

CHAPTER FIVE

General Discussion and Conclusion

Since Global Change is affecting the entire world at an alarmingly fast rate (IPCC, 2014) and entire ecosystems have been contaminated by fertilizers, pesticides and other xenobiotics in recent decades, we decided to conduct research considering these disturbances and investigating possible effects caused by them.

The question we aimed to answer was: how will Global Change including environmental pollution affect spring species? Switzerland is the "water castle of Europe" (http://www.swissworld.org/; Zollhöfer, 1999) and springs and headwaters are numerous. Unfortunately springs are not legally protected habitats in Switzerland (Zollhöfer, 1997) but are mostly thought of as sources of drinking water. It was assumed for a long time that springs were stable ecotones and as such not influenced by disturbances. However it has recently been shown that springs are no exception (von Fumetti et al., 2007). For example, the discharge of springs can vary and affect the distribution of macroinvertebrate assemblages (von Fumetti, 2008). Switzerland is a relatively small country surrounded by other land masses and is strongly influenced by the Alps, a mountain range with altitudes > 4800m. This is also one of the reasons why temperature warming, as a result of Global Change, has noticeable effects, as for example glaciers are melting (Clitherow et al., 2013; IPCC 2014). Thus important habitats are being lost or changed so dramatically that the species inhabiting those areas are forced to migrate. Migration into more suitable habitats is not always possible (Woodward et al., 2010) and so the extinction of certain highly specialised and endemic species will occur.

Ecotoxicological studies are usually conducted under strictly standardised conditions (Fent, 2013). We decided to follow another, much less frequently chosen approach and conducted our experiments as naturally as possible in flow channels. Although less standardised conditions inevitably lead to more variation in the results, we wanted to propagate a more natural approach in laboratory experiments. *G. fossarum* is subjected to daylight exposure in its natural environment and usually does not go through starvation periods. In our study the organisms were therefore

exposed to light and were fed. It was ensured that the test individuals were of similar size but we did not differentiate between sexes. A natural undisturbed population of gammarids consists of females and males, all of different age groups and sizes of organisms. Determination of the sex of the gammarids would have caused additional stress. The handling of the gammarids was kept to a minimum since we were interested in exposing the test organisms to as little stress as possible so that the results reflected, as far as possible, the temperature and copper influences.

It is known that the two chosen endpoints, the feeding and ETS activity, are influenced by a number of factors, while other endpoints such as growth or reproduction are also influenced by many factors. It is documented that the feeding activity of gammarids can be influenced by three factors: the tree species consumed (e.g. Maltby et al., 2002; Cold & Forbes, 2004; Bundschuh et al., 2009), the sex, as was shown for *G. pulex* (Malbouisson et al., 1994), and natural differences in appetites of individuals (Taylor et al., 1993). The ETS activity of organisms can be influenced by light (Simcic & Brancelj, 2007) and feeding or fasting (Mezek et al., 2010). Size influenced the ETS activity of the intertidal amphipod *Corophium volutator* (Pallas, 1766) (nomenclature according to Bousfield & Hoover, 1997) (Cammen et al., 1990). Other experiments have demonstrated that larger individuals of *G. fossarum* have a lower ETS activity (Simcic & Brancelj, 2003). The reason for this is the increasing proportion of metabolically inactive mass with increasing body size, since larger gammarids have a larger amount of metabolically inactive keratin protecting their bodies (Simcic & Brancelj, 2003). In a separate experiment we tested the effect of the sex of *G. fossarum* on the ETS activity and no significant differences were found. This is in line with findings that the ETS activity of crayfish was not affected by the sex but by temperature (Simcic et al., 2014).

Numerous experiments have been conducted with gammarids in the laboratory testing effects of different contaminants (e.g. Malbouisson et al., 1995; Bat et al., 2000; De Lange et al., 2006; Zubrod et al., 2010; Funck et al., 2013). However, very few studies consider water temperature and its effects on the exposed organisms. It has previously been shown that amphipods are able to tolerate acid stress for a longer period of time at lower temperatures than at higher

temperatures (Rinderhagen et al., 2000). *G. fossarum* has been shown to be stressed when high NaCl concentrations are coupled with high water temperatures (Georgiadis, 1977).

The experiments conducted in this thesis take temperature elevations and their impacts on the metabolism of the exposed gammarids into consideration. The results of our first set of experiments, in which water temperatures ranging from 10 to 18 °C were used, showed that *G. fossarum* is not able to tolerate water temperatures of 18 °C and mortality was accordingly very high. Many laboratory studies with gammarids use a single water temperature which usually ranges between 12 and 16 °C (e.g. Cold & Forbes, 2004; Bundschuh et al., 2009; Schaller et al., 2010; Coulaud et al., 2011). It has been shown that different lineages of *G. fossarum* differ in sensitivity towards xenobiotics (Feckler et al., 2012) and likewise we assume that they also have different temperature tolerances. The gammarids used in the tests of this project are naturally exposed to water with temperatures of about 10 °C all year round. Although an increase of 6 to 8 °C of water temperature is unlikely, we chose to test up to 18 °C to detect the temperature tolerance limit of this species. It is important to know the limiting factors when trying to protect a species. Mortality was highest at 18 °C and seems to be the tolerance limit of the gammarids used in this project. As expected the gammarids shredded more leaf material at the higher water temperatures, indicating a higher metabolic activity. This is in accordance with other studies about temperature influence on the metabolism of organisms (e.g. Nilsson, 1974; Georgiadis, 1977; Fent, 2013).

The heavy metal copper is essential for most organisms (e.g. Clarkson et al., 1991). It is an important ingredient in many fungicides (de Oliveira-Filho et al., 2004) but toxic when available in excess (De Martinez Gaspar Martins et al., 2011). For these reasons, and since it is so widely applied and even used in organic agriculture (e.g. Trewavas, 2004; Niggli, 2007), it was chosen as an additional stressor in our investigations. Copper ions are part of haemocyanin and therefore regulated in gammarid species (Taylor & Anstiss 1999). However, these regulation mechanisms are limited and so copper exposure also causes problems for gammarids such as *G. fossarum*. Temperature has often been quoted to have effects on the toxicity of pesticides and other pollutants (e.g. Fent, 2013), but most authors only conduct LC_{50}-tests at one temperature (e.g. Güven et al., 1999; Felten et al., 2008). In this project LC_{50}-tests with copper were conducted at water

temperatures ranging from 10 to 18 °C. Higher temperatures were shown to raise copper toxicity. Previous studies have been conducted on the effects of copper, for example on *G. pulex* (e.g. Taylor et al., 1998; Güven et al., 1999; Brooks & Mills, 2003), *G. duebenii* Liljeborg, 1851 (nomenclature according to own nomenclatorial investigations) (e.g. Lawrence & Poulter, 1998) and *G. aequicauda* (Martynov, 1931) (nomenclature according to own nomenclatorial investigations) (e.g. Prato et al., 2013), but not many have been done with *G. fossarum*. Dedourge-Geffard et al. (2009) studied the effects of inter alia copper on the feeding rate and digestive enzymes of *G. fossarum* and found that organisms originating from a metallic contaminated site showed inhibited digestive enzymes and a decreased feeding activity. In accordance with this, we showed that copper ions tended to reduce feeding activity and significantly lowered the ETS activity of the organisms. These effects were noted in flow channels in the laboratory as well as in the spring. Furthermore these effects were found when the copper ions were in the water or on the leaf litter.

The results of the experiments conducted in this project help us to answer the question of how gammarids will respond to the ongoing Global Change, including environmental pollution. Given that the water quality is not impaired, we conclude that a temperature increase of about two degrees will not endanger *G. fossarum*. If, however, an additional stressor such as copper is added, then a water temperature elevation will not be tolerated. Furthermore we conclude that spring species which are more sensitive towards temperature will not be able to tolerate temperature elevations as easily, especially when considering all the other influences to which they are exposed. It can be concluded that *G. fossarum* is a useful model organism for freshwater research in combination with toxic substances and Global Change. In addition the chosen approach of more natural conditions in the laboratory was helpful in assessing the effects of temperature and copper ions. Lastly, the experimental set-up with test chambers proved promising for future field experiments in springs to test effects of contaminated food sources. The use of test chambers, as we designed them, could also become an important tool for biomonitoring of springs and other larger freshwaters.

References

Bat L, Akbulut M, Culha M, Gündogdu A, Satilmis HH (2000). Effect of Temperature on the Toxicity of Zinc, Copper and Lead to the Freshwater Amphipod *Gammarus pulex pulex* (L., 1758). Turkish Journal of Zoology 24: 409-415.

Bundschuh M, Hahn T, Gessner MO, Schulz R (2009) Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 28: 197-203.

Brooks SJ, Mills CL (2003) The effect of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. Comparative Biochemistry and Physiology Part A 135: 527-537.

Bousfield EL, Hoover PW (1997) The amphipod superfamily Corophioidea on the Pacific coast of North America: 5. Family Corophiidae: Corophiinae, new subfamily: systematics and distributional ecology. Amphipacifica 2:3:67-139

Cammen LM, Corwin S, Christensen JP (1990) Electron transport system (ETS) activity as a measure of benthic macrofaunal metabolism. Marine Ecology Progress Series 65:171-182.

Merian (ed.) (1991) Metals and Their Compounds in the Environment. Occurence, Analysis, and Biological relevance. Edited by Merian E. VCH Verlagsgesellschaft mbH, Weinheim.

Clitherow LR, Carrivick JL, Brown LE (2013) Food Web Structure in a Harsh Glacer-Fed River. PLOS ONE 8:4: e60899.

Cold A, Forbes VE (2004) Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*. Aquatic Toxicology 67: 287-299.

Coulaud R, Geffard O, Xuereb B, Lacaze E, Quéau H, Garric J, Charles S, Chaumot A (2011) In situ feeding assay with *Gammarus fosssarum* (Crustacea): Modelling the influence of confounding factors to improve water quality biomonitoring. Water Research 45: 6417-6429.

De Lange HJ, Noordoven W, Murk AJ, Lürling M, Peeters ETHM (2006) Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquatic Toxicology 78: 209-216.

De Martinez Gaspar Martins M, Barcarolli IF, De Menezes EJ, Giacimin MM, Wood CM, Bianchini A (2011) Acute toxicity, accumulation and tissue distribution of copper in the blue crab *Callinectes sapidus* acclimated to different salinities: *In vivo* and *in vitro* studies. Aquatic Toxicology 101: 88-99.

De Oliveira-Filho EC, Lopes RM, Paumgartten FJR (2004) Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56: 369-374.

Dedourge-Geffard O, Palais F, Biagianti-Risbourg, Geffard O, Geffard A (2009) Effects of metals on feeding rate and digestive enzymes in *Gammarus fossarum*: An *in situ* experiment. Chemosphere 77: 1569-1576.

Feckler A, Thielsch A, Schwenk K, Schulz R, Bundschuh M (2012) Differences in the sensitivity among cryptic lineages of the *Gammarus fossarum* complex. Science of the Total Environment 439: 158-164.

Felten V, Charmantier G, Mons R, Geffard A, Rousselle P, Coquery M, Garric J, Geffard O (2008) Physiological and behavioural responses of *Gammarus pulex* (Crustacea: Amphipoda) exposed to cadmium. Aquatic Toxicology 86: 413-425.

Fent K (2013) Ökotoxikologie. Fourth Edition. Thieme Verl., Stuttgart, New York.

Funck JA, Danger M, Gismondi E, Cossu-Leguille C, Guérold F, Felten F (2013) Behavioural and physiological responses of *Gammarus fossarum* (Crustacea Amphipoda) exposed to silver. Aquatic Toxicology 142-143: 73-84.

Georgiadis G (1977) Die Auswirkung verschiedener Temperatur- und Salinitätskombinationen auf *Gammarus fossarum* Koch, 1835. Crustaceana Supplement, Number 4, Studies in Gammaridae (Proceedings of the 3rd International Colloquium on Gammarus and Niphargus, Schlitz, 1975), 112-119.

Güven K, Özbay C, Ünlü E, Satar A (1999) Acute Lethal Toxicity and Accumulation of Copper in *Gammarus pulex* (L.) (Amphipoda). Turkish Journal of Biology 23: 513-521.

http://www.swissworld.org/de/geografie/fluesse_und_seen/ Die Quellen Europas liegen in der Schweiz. Last visited on 4th February 2015.

IPCC 2014. Climate Change (2014) Synthesis Report. Longer Report. IPCC Fifth Assessment Synthesis Report. 116 pages. Last visited on 4th February 2015: http://www.ipcc.ch/pdf/assessment-report/ar5/syr/SYR_AR5_LONGERREPORT_Corr2.pdf.

Lawrence AJ, Poulter C (1998) Development of a sub-lethal pollution bioassay using the estuarine amphipod *Gammarus duebeni.* Water research 32: 569-578.

Malbouisson JFC, Young TWK, Bark AW (1994) Disruption of Precopula in *Gammarus Pulex* as a Result of Brief Exposure to Gamma-Hexachlorocyclohexane (Lindane). Chemosphere 28: 2011-2020.

Malbouisson JFC, Young TWK, Bark AW (1995) Use of feeding rate and repairing of precopulatory *Gammarus pulex* to assess toxicity of Gamma-Hexachlorocyclohexane (Lindane). Chemosphere 30: 1573-1583

Maltby L, Clayton SA, Wood RM, McLoughlin N (2002) Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: Robustness, responsiveness, and relevance. Environmental Toxicology and Chemistry 21:2: 361-368.

Mezek T, Simcic T, Arts MT, Brancelj A (2010) Effect of fasting on hypogean (*Niphargus stygius*) and epigean (*Gammarus fossarum*) amphipods: a laboratory study. Aquatic Ecology 44: 397-408.

Niggli U (2007) Mythos "bio". Kommentare zum gleichnamigen Artikel von Michael Miersch in der Wochenzeitung „Die Weltwoche" vom 20. September 2007. Last visited on 4th February 2015: http://orgprints.org/11368/1/niggli-2007argumentarium.pdf

Nilsson LM (1974) Energy Budget of a Laboratory Population of *Gammarus pulex* (Amphipoda). OIKOS 25:1: 35-42.

Prato E, Parlapiano I, Biandolino F (2013) Sublethal effects of copper on some biological traits of the amphipod *Gammarus aequicauda* reared under laboratory conditions. Chemosphere 93:6: 1015-1022.

Rinderhagen M, Ritterhof J, Zauke G (2000) Crustaceans as Bioindicators. From: Biomonitoring of Polluted Water - Reviews on Actual Topics (A. Gerhardt, ed.), Trans Tech Publications - Scitech Publications, Environmental Research Forum 9:161-194.

Schaller J, Mkandawire M, Dudel EG (2010) Heavy metals and arsenic fixation into freshwater organic matter under *Gammarus pulex* L. influence. Environmental Pollution 158: 2454-2458.

Simcic T, Brancelj A (2003) Estimation of the proportion of metabolically active mass in the amphipod *Gammarus fossarum*. Freshwwater Biology 48: 1093-1099.

Simcic T, Brancelj A (2007) The effect of light on oxygen consumption in two amphipod crustaceans – the hypogean *Niphargus stygius* and the epigean *Gammarus fossarum*. Marine and Freshwater Behaviour and Physiology 40:2: 141-150.

Simcic T, Pajk F, Jaklic M, Brancelj A, Vrezec A (2014) The thermal tolerance of crayfish could be estimated from respiratory electron transport system activity. Journal of Thermal Biology 41:21-30.

Taylor HH, Anstiss JM (1999) Copper and haemocyanin dynamics in aquatic invertebrates. Marine and Freshwater Research 50:8: 907-931.

Taylor EJ, Jones DPW, Maund SJ, Pascoe D (1993) A New Method For Measuring the Feeding Activity of *Gammarus Pulex* (L.). Chemosphere 26: 1375-1381.

Taylor EJ, Underhill KM, Blockwell SJ, Pascoe D (1998) Haem biosynthesis in the freshwater macroinvertebrate *Gammarus pulex* (L.): Effects of copper and Lindane. Water Research 32: 2202-2204.

Trewavas A (2004) Review: A critical assessment of organic farming-and-food assertions with particular respect to the UK and the potential environmental benefits of no-till agriculture. Crop Protection 23: 757-781.

Von Fumetti S (2008) Dissertation: Distribution, discharge and disturbance: new insights into faunal spring ecology. Universität Basel, Basel.

Von Fumetti S, Nagel P, Baltes B (2007) Where a springhead becomes a springbrook - a regional zonation of springs. Fundamental and Applied Limnology 169:1: 37-48.

Woodward G, Perkins DM, Brown LE (2010) Climate change and freshwater ecosystems; impacts across multiple levels of organization. Philosophical transactions of the royal society B 365: 2093-2106.

Zollhöfer JM (1997) Quellen - die unbekannten Biotope im Schweizer Jura und Mittelland. Erfassen - Bewerten - Schützen. Bristol Stiftung, Zürich.

Zollhöfer JM (1999) Spring Biotopes in Northern Switzerland: Habitat Heterogeneity, Zoobenthic Communities and Colonization Dynamics.Dissertation. ETH Zürich, Zürich.

Zubrod JP, Bundschuh M, Schulz R (2010) Effects of subchronic fungicide exposure on the energy processing of Gammarus fossarum (Crustacea; Amphipoda). Ecotoxicology and Environmental Safety 73: 1674-1680.

SUMMARY

Global Change including climate change, environmental pollution and habitat destruction are taking place all over the world. Temperature rises are occurring and changing the ecosystems. Glaciers are melting and as a result the sea level is rising, just to give one example of the impact Global Change is having. Taking these changes into consideration and the fact that little is known about the exact consequences of these changes on freshwater species, the aim of this thesis was to find out how a model organism reacts to rising water temperatures and copper exposure. In order to test this, experiments were conducted with *G. fossarum* in flow channels in a laboratory and one set of experiments was conducted in the field directly in a spring.

In the first set of experiments the temperature tolerance of *G. fossarum* was determined in laboratory flow channel experiments. Three different temperature scenarios were tested and the end points chosen in these and all further experiments were the feeding and respiratory electron transport system (ETS) activity. These endpoints are complementary in determining different aspects of the metabolic activity of the tested organisms. The feeding activity increased with temperature increase; no significant temperature effect on the ETS activity was observed. This is discussed and the implications of our results portrayed for more sensitive spring species.

In the second set of experiments an additional stressor in the form of copper sulphate was added to the set-up, in order to assess what impact elevated water temperatures in combination with a sub-lethal copper exposure would have on *G. fossarum*. Although it is known that temperature can raise the toxicity of substances and is a determining factor for growth, relatively few experiments have been conducted in the field of ecotoxicology considering temperature. Therefore we conducted a set of LC_{50}-tests at different water temperatures prior to these experiments. We were able to confirm that elevated water temperature raises copper toxicity to gammarids. The second set of experiments demonstrated no significant effect of copper on the feeding activity; it was however slightly raised at the higher water temperatures. The ETS activity of *G. fossarum* was significantly lowered when exposed to copper, but increased with increasing water temperature. In this set of experiments we demonstrated the importance of using different endpoints to find

answers to a question. The approach of using two methods which enable assertions on the same biological responses is desirable. A higher risk of adverse effects with increase in water temperature and exposure to copper can be reasonably inferred from our results.

The value of field experiments is not disputed and yet experiments of such nature are seldom, especially in the field of ecotoxicology. Flow channel experiments are a good option for conducting experiments with freshwater species under controlled conditions. In this project we went a step further and conducted the stird set of experiments in the natural habitat of *G. fossarum*. We designed the experimental set-up to be suitable for experiments both in flow channels in the laboratory and in a natural spring. Since we did not want to pollute the entire spring we opted for contaminated leaf litter, which was placed in the spring in test chambers. The water temperature was not changed in this set of experiments. The feeding activity was not significantly affected by the copper; the ETS activity was significantly lowered. Generally the metabolic activity of the gammarids was higher in the laboratory than in the spring. In this last set of experiments we took a crucial step towards a more realistic approach when examining environmental pollutants on organisms.

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