



Article

Heat Tolerance of *Gammarus fossarum* (Crustacea, Amphipoda) Is Influenced by the Level of Stress Associated with Reproduction and the Water Quality of Their Habitat

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Abstract: To elucidate how rising temperatures influence native amphipods, and how the expansion of wastewater treatment plants (WWTPs) with a fourth purification stage can alter temperature-related effects, studies were conducted in anthropogenically influenced rivers in Southwest Germany. Gammarids were sampled up- and downstream of two WWTPs, and exposed to elevated temperatures in the laboratory. Gammarids separated from precopula pairs were more sensitive to exposure-related stress and to a temperature increase than animals that were not in the reproductive phase. Furthermore, females were significantly more sensitive than males. Adult gammarids sampled individually at the WWTP with three purification stages showed a similar tolerance to elevated temperature at the up- and downstream site. In contrast, mortality following heat stress was substantially lower in animals sampled downstream of the WWTP with four treatment stages than in those sampled at the upstream site. We assume that the increased sensitivity of artificially separated precopula pairings reflect the situation under high hydraulic stress after heavy rainfall. Our results further illustrate the importance of WWTP upgrades in the context of climate change: gammarids sampled downstream the upgraded fourth-stage WWTP were likely exposed to lower levels of micropollutants, and thus may have allocated energy from detoxification to heat stress responses.

Keywords: gammarids; multiple stress; heat tolerance; wastewater treatment plants; climate change



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1. Introduction

Today, our rivers and streams are not only affected by the release of micropollutants from wastewater treatment plants, stormwater overflows, and agricultural areas. Over the last decade, other stressors on aquatic ecosystems have been increasingly identified [1]. Climate change has already led to prolonged heat waves and heavy rain events worldwide, which are expected to increase in duration and intensity [2]. As a result of more dry periods, significantly increased proportions of treated effluent from wastewater treatment plants (WWTPs), higher water temperatures, and lower oxygen levels during dry weather periods [3–5] are now contrasted with the temporary intake of high volumes of untreated wastewater due to stormwater overflow basin (SOB) discharges, agricultural run-off, the remobilization of pollutants from sediment, particulate loads, and hydraulic stress during heavy rain events [6–8]. Globally, an average increase in water temperatures of at least 0.5 °C per decade has already been observed [9–12]. Temperature projections indicate that water temperatures will increase by up to 4 °C by 2050 if climate change continues unabated [13].

With regards to organic pollution by nutrients, the ecological status of running waters has improved significantly in the last decades, mainly due to the improved nutrient

reduction in wastewater treatment plants [14–18]. However, this contrasts with the dramatic decline in biodiversity in freshwater ecosystems, where biodiversity loss is more pronounced than anywhere else [19–23]. One reason for this might be, in addition to influences of temperature and structural deficiencies, that rivers are constantly exposed to a large number of different micropollutants that enter aquatic ecosystems through various pathways and can cause adverse effects in exposed organisms [24–26]. The addition of a fourth treatment stage to wastewater treatment plants (WWTPs) has proven to be an effective tool to reduce the discharge of micropollutants from these installations. In recent years, there has been a significant increase in the number of studies demonstrating the positive effects of the use of ozonation or powdered activated carbon in WWTPs on aquatic ecosystems [27–34].

It is undisputed that climate change will affect aquatic ecosystems and, in particular, their organisms. As a result of hotter and longer summers and the resulting drier weather periods, water levels in rivers and streams will decrease drastically and will further increase the concentration of micropollutants. Whenever wastewater discharged from treatment plants contains high concentrations of micropollutants that cannot be diluted by the receiving water body due to low water levels or pre-contamination from various sources that has already occurred upstream, the importance of upgrading wastewater treatment plants with a fourth treatment stage to further minimize the input of micropollutants becomes apparent. This may help reduce the chemical impact of rising temperatures on river ecosystems.

In this study, we investigated the relationship between water quality, which is influenced by different ways of wastewater treatment, and the heat tolerance of amphipods as an important aspect of the adaptive capacity of aquatic organisms to climate change. For this purpose, we exposed individuals of *Gammarus fossarum* of different life stages, collected from two anthropogenically influenced rivers up- and downstream of differently equipped WWTPs to water from the respective sampling sites at two different temperatures in the laboratory. As most studies on gammarids have examined the effects of selected (toxic) substances in combination with heat stress under controlled laboratory conditions [35–38], this study is, to our knowledge, the first study with an explicit reference to outdoor situations that examine heat stress as a confounding factor for gammarids sampled upstream and downstream of differently equipped WWTPs.

2. Materials and Methods

2.1. Sampling Sites

Gammarids were sampled at two tributaries to the Neckar river in Southwestern Germany, the rivers Erms and Eyach. *Gammarus fossarum* is naturally present in the river sections investigated in the present study. No other gammarid species are found there. At the river Erms (Figure 1), the sampling took place up- and downstream of the wastewater treatment plant Bempflingen-Riederich (“3-stage-WWTP”), which is the second WWTP connected to the river Erms in the direction of the flow. Its size is designed for a population equivalent of 30,000, and it is equipped with three treatment stages (mechanical and chemical–biological wastewater treatment; for discharge characteristics of this WWTP see Supplemental Table S1). Sampling site “3-stage-WWTP_{upstream}” was located 1.5 km upstream of the 3-stage-WWTP (N 48°34′6.24″, E 9°15′44.64″), whereas site “3-stage-WWTP_{downstream}” was located directly downstream of the 3-stage-WWTP (N 48°34′50.5″, E 9°15′29.9″). At the river Eyach (Figure 1), sampling sites were also selected up- and downstream of a WWTP, in this case, the WWTP Albstadt-Lautlingen (“4-stage-WWTP”). This WWTP is designed for a population equivalent of 36,000 and is equipped with four treatment stages, including a powdered activated carbon stage which was installed in 1992 (for discharge characteristics of the 4-stage-WWTP, see Supplemental Table S2). The sampling site “4-stage-WWTP_{upstream}” was located 250 m upstream of the 4-stage-WWTP (N 48°12′53.64″, E 8°56′33.36″), and site “4-stage-WWTP_{downstream}” was located directly downstream of the 4-stage-WWTP (N 48°12′55.08″, E 8°56′21.84″).

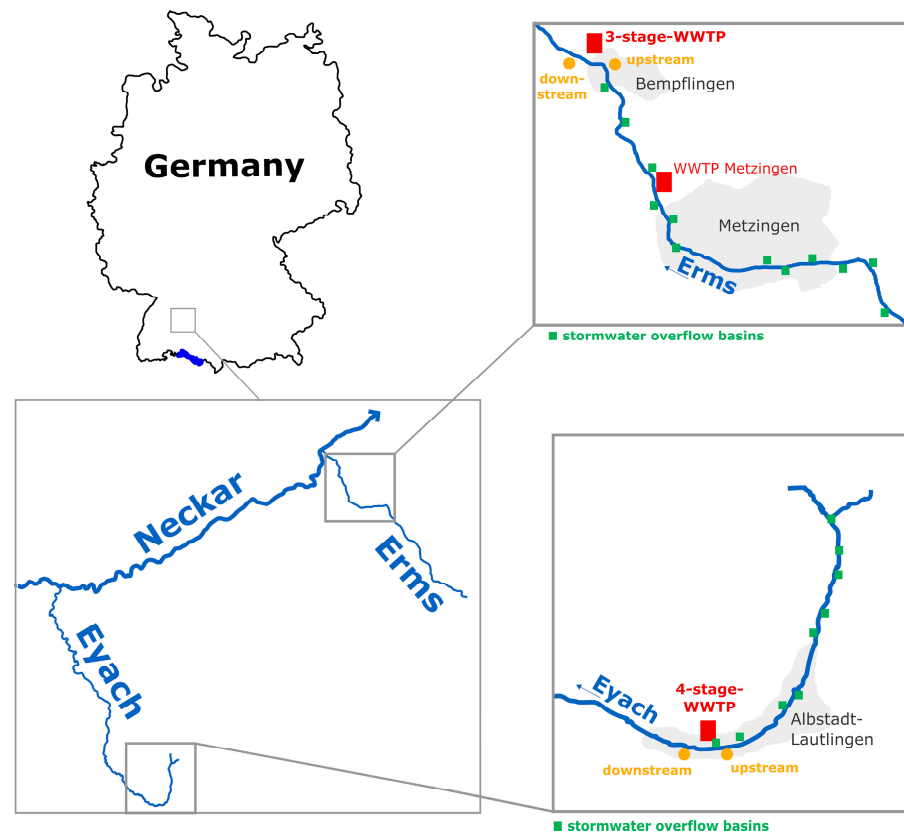


Figure 1. Location of sampling sites. Sampling took place at the river Erms, upstream and downstream of the wastewater treatment plant Bempflingen-Riederich (3-stage-WWTP). Further wastewater treatment plants (WWTPs) and stormwater overflow basins (SOBs) connected to the Erms are marked in red (WWTP Metzingen) and green (SOBs). At the Eyach river, sampling sites are also located upstream and downstream of the WWTP Albstadt-Lautlingen (4-stage-WWTP). SOBs connected to the river Eyach are marked in green.

2.2. Exposure of Gammarids

At each sampling site, gammarids were pick-sampled at typical habitats such as roots, macrophytes, and conglomerations of leaves, using a conventional sieve (with a mesh size of 1 mm). Water containers (capacity 15 L) were used to transport the organisms to the laboratory. In addition, 15 L of water was separately collected from each sampling site and also transported to the lab. All experiments took place under controlled conditions in climate chambers. The exposure of gammarids was performed at two different temperatures: (a) under normal ambient conditions (control temperature equaling the temperature of the rivers at the time of sampling) and (b) under heat stress at 25 °C symbolizing extreme environmental warming conditions. The optimum temperature for *Gammarus fossarum* is between 12 °C and 20 °C [39]. Temperatures below 12 °C are tolerated, but fecundity, reproduction, and hatching are drastically reduced [40,41]. Temperatures above 24 °C are considered as heat stress for *G. fossarum* in the literature [39–41], so we chose 25 °C as a heat stress condition in our experiments. An illumination regime of 14 h of light and 10 h of darkness was set in both climate chambers. Gammarids were kept in a 10 L glass aquaria and filled with 5 L of constantly aerated water from each sampling site. Two stainless steel grids (7 × 20 cm, mesh size 1 mm, accordion folded), placed in each aquarium, served as hiding places. Cotton compresses, pre-conditioned in water of the respective sample site for one week, served as food. The time of exposure in both experiments was one week. No water exchanges were performed during this time. Physicochemical water parameters were recorded at the beginning and at the end of exposure.

2.2.1. Exposure of Male and Female Gammarids Separated from Precopula Pairs

In July 2020, gammarids were sampled as precopula pairs at each sampling site. Pairs were manually separated on-site, and males and females were stored in separate transport containers. Fifty organisms of each sex were placed in separate aquaria and exposed to 15 °C (control temperature) and 25 °C (heat stress) for one week. Due to the restricted availability of precopula pairs, the number of animals used was limited. As we expected a high mortality during heat stress, we wanted to run the experiment with a sufficiently high number of individuals per aquarium. Consequently, the experiment could not be carried out in a three-block design. Accordingly, eight aquaria (male, female, upstream and downstream WWTP, and Eyach and Erms) were set up under control and heat stress conditions, with 50 individuals each (total sample size: 800 gammarids). Gammarid mortality was recorded at the end of the exposure.

2.2.2. Exposure of Gammarids of Unknown Sex

In August 2020, individual gammarids were sampled at each sampling site and 50 organisms were randomly placed in each aquarium, independent of their sex, as sexing is not possible in the field with this species. Gammarids were exposed to 18 °C (control temperature) and 25 °C (heat stress) for one week. In order to be able to compare this experiment with the one above, we kept the setup with gammarids of unknown sex exactly the same as in the experiment with precopula pairs. Accordingly, four aquaria (upstream and downstream WWTP; Eyach and Erms) were set up under control and heat stress conditions, with 50 individuals each (total sample size: 400 gammarids). At the end of the exposure, gammarid mortality was recorded and the sex of surviving individuals was determined according to Eggers and Martens [42].

2.3. Sex Ratio in Field Samples

Except at the precopula stage, sex determination in the field is not possible in living individuals of *G. fossarum*. In August 2020, gammarids were exposed without knowing their sex at the beginning of the exposure; this was determined at the end of the experiment. However, in order to obtain realistic information of the animals' sex ratio at the start of exposure, randomly collected individuals per field site were sexed. For this purpose, 250 gammarids were randomly sampled at each sampling site on typical habitats such as roots, macrophytes, and leaf assemblages using a conventional sieve (mesh size: 1 mm) and fixed in 70% ethanol on-site. The species and sex of each individual were determined by external morphological characteristics according to Eggers and Martens [42]. Female gammarids were characterized by four pairs of oostegites (brood plates) and males by two genital papillae on the ventral body side [43]. Since external sexual organs are not developed until the ninth molt, small gammarids (body length < 6 mm) were defined as "juvenile" [39]. As sampling for the second exposure experiment also took place on a random basis, we assume the sex ratio of exposed gammarids at the start of the experiment to be identical to the sex ratio found at the respective field sites. At the end of the exposure, the sex of the surviving gammarids was determined and compared to the sex ratio obtained in the field samples. If one of the sexes were more sensitive during the exposure, the sex ratio at the end of the exposure would have shifted and thus would have differed from that found in the field.

2.4. Physicochemical Parameters

Water pH, oxygen concentration, conductivity, and temperature were measured directly in each aquarium at the beginning and at the end of exposure, and in the field when sampling was performed. In additional water samples taken from each aquarium, the concentrations of nitrate, nitrite, ammonium, phosphate, and chloride were determined photometrically (NANOCOLOR® tube tests and compact photometer PF-12Plus, Macherey-Nagel, Düren, Germany), whereas carbonate and total hardness were determined titrimetrically (MQuant®, Merck, Darmstadt, Germany).

2.5. Data Analyses and Statistics

The statistical analyses were conducted with JMP® 16.0.0 (SAS Institute Inc., Cary, NC, USA). The mortality data of both experiments as well as the distribution of sex in the field were assessed with a likelihood-ratio χ^2 test, followed by pairwise Fisher's exact tests. The α -levels were corrected for multiple testing according to the Benjamini–Hochberg method [44].

3. Results

3.1. Exposure of Gammarids

3.1.1. Exposure of Male and Female Gammarids Separated from Precopula Pairs

(a). Exposure to the water temperature prevailing at the time of sampling, 15 °C

With the exception of females collected in the Eyach river (sampling sites “4-stage-WWTP_{upstream}” and “4-stage-WWTP_{downstream}”), the mortality of male and female gammarids exposed to the control temperature (15 °C) was less than 20%, showing a slight trend toward higher mortality at upstream sampling sites compared to downstream sites of both WWTPs (Figure 2). Also, females' mortality was higher than males' mortality, both upstream and downstream of both wastewater treatment plants. Downstream of the 4-stage-WWTP, this difference was statistically detectable (Fisher's exact test, $df = 1$, $n = 100$, $p = 0.0019$). Comparing the two rivers, the mortality of both sexes was higher up- and downstream of the 4-stage-WWTP than at the up- and downstream sampling sites of the 3-stage-WWTP. The mortality of females downstream of the 4-stage-WWTP was significantly higher than that of females downstream of the 3-stage-WWTP (Fisher's exact test: $df = 1$, $n = 100$, $p = 0.005$).

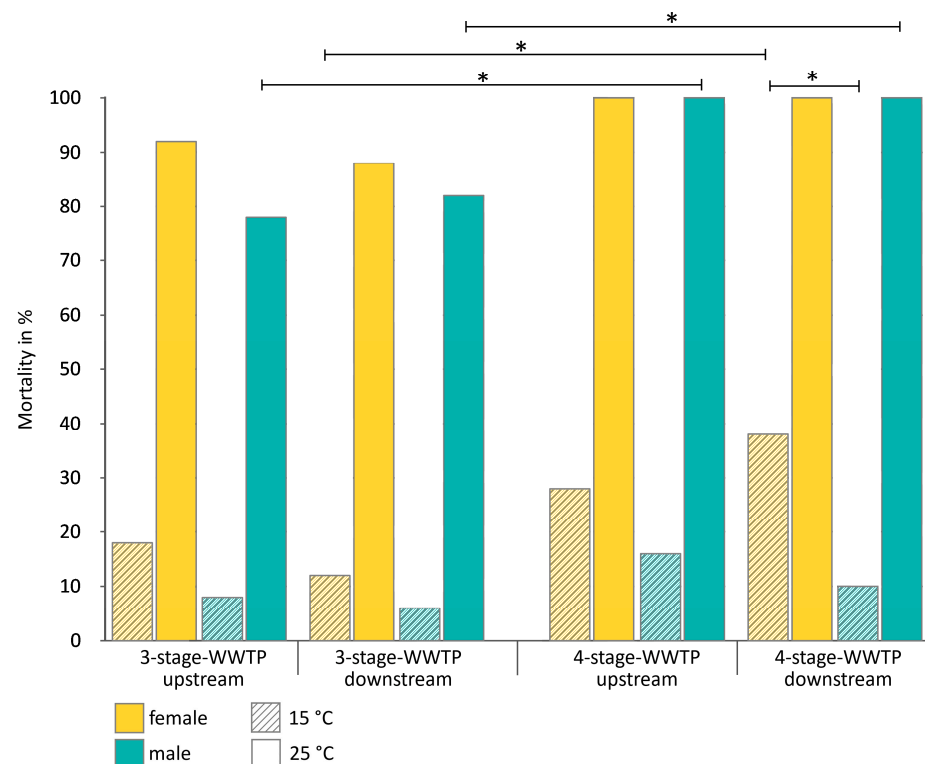


Figure 2. Mortality of female (in yellow) and male (in teal) gammarids (*Gammarus fossarum*, separated from precopula pairs) from sampling sites up- and downstream of the 3-stage- and 4-stage-WWTP, exposed to 15 °C (control temperature, shaded) and 25 °C (heat stress, unshaded) in July 2020. 3-stage-WWTP: equipped with three treatment stages; 4-stage-WWTP: equipped with four treatment stages (including activated carbon). Sample size $n = 50$ for all treatments. Significant differences (Fisher's exact test following by Benjamini–Hochberg correction) within the single temperature approaches are marked with asterisks * (15 °C: 4-stage-WWTP_{downstream} female vs. 4-stage-WWTP_{downstream} male: $p = 0.0019$; 25 °C: 3-stage-WWTP_{upstream} female vs. 3-stage-WWTP_{downstream} female: $p = 0.005$; 3-stage-WWTP_{upstream} female vs. 3-stage-WWTP_{downstream} male: $p = 0.005$; 4-stage-WWTP_{upstream} female vs. 4-stage-WWTP_{downstream} female: $p = 0.005$; 4-stage-WWTP_{upstream} female vs. 4-stage-WWTP_{downstream} male: $p = 0.005$).

$df = 1, n = 100, p \leq 0.0019$; 3-stage-WWTP_{downstream} female vs. 4-stage-WWTP_{downstream} female: $df = 1, n = 100, p \leq 0.005$. 25 °C: 3-stage-WWTP_{upstream} male vs. 4-stage-WWTP_{upstream} male: $df = 1, n = 100, p \leq 0.0005$; 3-stage-WWTP_{downstream} male vs. 4-stage-WWTP_{downstream} male: $df = 1, n = 100, p \leq 0.0026$). For clarity, the significant differences between the treatments at different temperatures are not explicitly displayed in the graph: in all cases, mortality was significantly higher at 25 °C than at 15 °C for both females and males, upstream and downstream of both WWTPs (Fisher's exact test: $df = 1, n = 100, p \leq 0.001$).

(b). Exposure to temperature extremes at 25 °C

After one week of exposure at 25 °C, all male and female gammarids died both up- and downstream of the 4-stage-WWTP, and showed a higher mortality than organisms up- and downstream of the 3-stage-WWTP (mortality between 78% and 92%) (Figure 2). In male gammarids, this difference was even statistically significant. Concerning the upstream sampling sites, males' mortality was significantly higher upstream of the 4-stage-WWTP compared to upstream of the 3-stage-WWTP (Fisher's exact test: $df = 1, n = 100, p = 0.0026$). The same was true for the downstream sampling sites: males' mortality was significantly higher downstream of the 4-stage-WWTP than downstream of the 3-stage-WWTP (Fisher's exact test: $df = 1, n = 100, p = 0.0005$).

Comparing the two temperatures, it is evident that gammarids were generally much more affected at 25 °C (environmental warming) than at 15 °C (control temperature), as mortality was significantly higher at 25 °C than at 15 °C for both females and males, upstream and downstream of both WWTPs (Fisher's exact test: $df = 1, n = 100, p \leq 0.001$), but mortality was generally higher in animals collected up- and downstream of the 4-stage-WWTP.

3.1.2. Exposure of Gammarids of Unknown Sex

(a). Exposure to the water temperature prevailing at the time of sampling, 18 °C

After one week of exposure to a control temperature of 18 °C, the gammarid mortality was less than 6% at all sampling sites (Figure 3). There was no difference in mortality between the upstream and downstream sites of each WWTP (3-stage-WWTP_{upstream} vs. 3-stage-WWTP_{downstream}; 4-stage-WWTP_{upstream} vs. 4-stage-WWTP_{downstream}) or between the two WWTPs (3-stage-WWTP_{upstream} vs. 4-stage-WWTP_{upstream}; 3-stage-WWTP_{downstream} vs. 4-stage-WWTP_{downstream}).

(b). Exposure to temperature extremes at 25 °C

At all sampling sites, an increase in temperature to 25 °C resulted in increased gammarid mortality after one week (Figure 3). Compared to the exposure of male and female gammarids separated from precopula pairs in the first experiment, where mortality was above 76% at all sampling sites, mortality in the present experiment was considerably lower. Mortality was between 54% and 62% at the sampling sites up- and downstream of the 3-stage-WWTP. Upstream of the 4-stage-WWTP, mortality was similar at 54%. The lowest mortality was found downstream of the 4-stage-WWTP (30%).

Similar to the first experiment (Figure 2), the mortality of the—here unsexed—gammarids was also significantly higher at the elevated temperature (25 °C) than at the control temperature (18 °C). This was the case for both WWTPs, both upstream and downstream of the respective WWTP (Fisher's exact Test: $df = 1, n = 100, p \leq 0.0001$). However, at the 4-stage-WWTP, a clear difference can be observed between the sampling sites upstream and downstream. The mortality at 4-stage-WWTP_{downstream} (30%) was significantly lower than at 4-stage-WWTP_{upstream} (54%) (Fisher's exact Test: $df = 1, n = 100, p \leq 0.0253$).

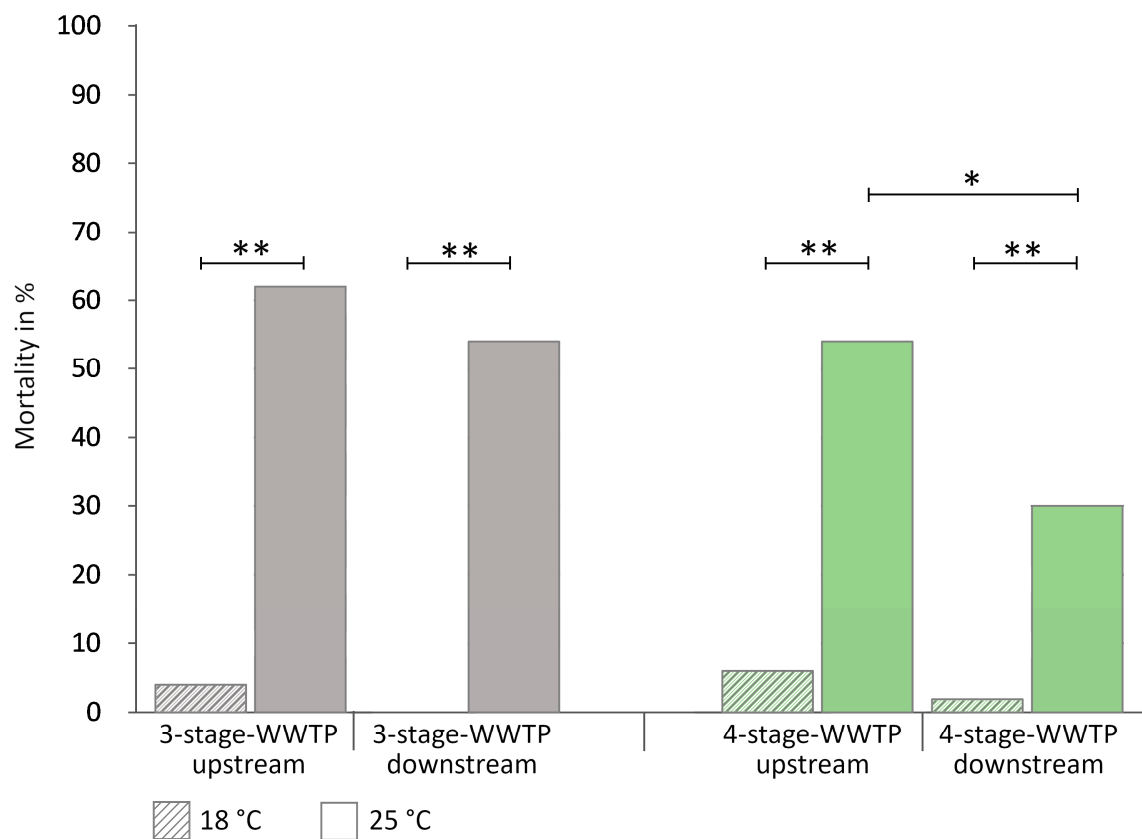


Figure 3. Mortality of gammarids (*Gammarus fossarum*) of unknown sex from sampling sites up- and downstream of the 3-stage-WWTP (in grey) as well as up- and downstream of the 4-stage-WWTP (in green), exposed to 18 °C (control temperature, shaded) and 25 °C (heat stress, unshaded) in August 2020. 3-stage-WWTP: equipped with three treatment stages; 4-stage-WWTP: equipped with four treatment stages (including activated carbon). The sample size is always $n = 50$. Significant differences (Fisher's exact test following by Benjamini-Hochberg correction) are marked with asterisks (** $df = 1$, $n = 100$, $p \leq 0.0001$; * $df = 1$, $n = 100$, $p \leq 0.0253$).

3.2. Determination of Sex Ratio (Random Field Samples vs. Exposed Gammarids in August 2020)

For randomly collected gammarids in the field, the proportion of males ranged from 50 to 60% at all sampling sites (Figure 4). At the end of the experiment, the sex ratio of gammarids exposed to the control temperature was not significantly different from the sex ratio of the “field” gammarids in any of the treatments. Similarly, no significant differences in the sex ratio were observed between the sampling sites at 18 °C. There were also no significant differences in sex ratios compared to field gammarids or between sampling sites when comparing animals exposed to 25 °C. Both sexes responded to stress with equal sensitivity.

3.3. Physicochemical Parameters

The limnological parameters measured during gammarid sampling showed only minor differences between the two sampling campaigns (for details, see Supplemental Table S3). For the river connected to the 4-stage-WWTP, data largely indicated a very good ecological status according to the German surface waters regulation (“Oberflächengewässerverordnung” [45]). Only the levels of orthophosphate can be considered to be in good ecological status in both July and August 2020 ($\text{PO}_4\text{-P}$ concentrations ≤ 0.05 mg/L). Another noticeable value was the higher concentration of chloride in July 2020 downstream of the 4-stage-WWTP (71 mg/L vs. 36 mg/L upstream of the 4-stage-WWTP), which did not occur again in August 2020 (36 mg/L upstream of the 4-stage-WWTP vs. 38 mg/L downstream of the 4-stage-WWTP).

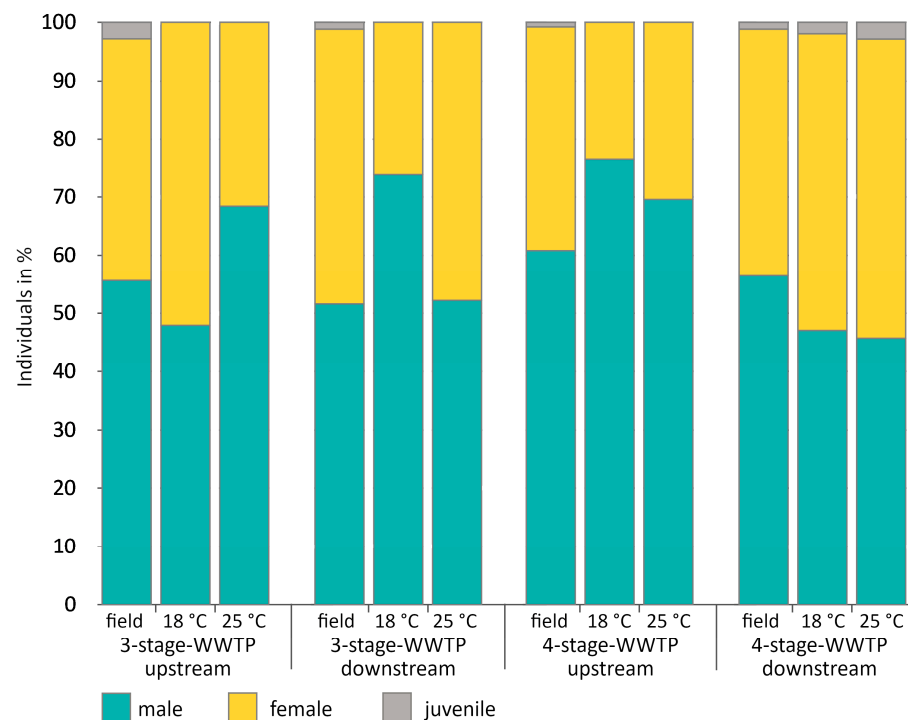


Figure 4. Percentage of male (in teal), female (in yellow), and juvenile (in grey) gammarids (*Gammarus fossarum*) directly at the sampling sites (field) up- and downstream of the 3-stage-WWTP and 4-stage-WWTP as well as after one week of exposure at 18 °C (control temperature) and 25 °C (heat stress). 3-stage-WWTP: equipped with three treatment stages; 4-stage-WWTP: equipped with four treatment stages (including activated carbon). The sample size in field samples is always $n = 250$. Sample size in the exposure experiment after one week: 3-stage-WWTP_{upstream} 18 °C = 48; 3-stage-WWTP_{upstream} 25 °C = 19; 3-stage-WWTP_{downstream} 18 °C = 50; 3-stage-WWTP_{downstream} 25 °C = 23; 4-stage-WWTP_{upstream} 18 °C = 47; 4-stage-WWTP_{upstream} 25 °C = 23; 4-stage-WWTP_{downstream} 18 °C = 51; 4-stage-WWTP_{downstream} 25 °C = 35.

In the river connected to the 3-stage-WWTP, however, several parameters indicate only a good ecological status according to the German surface waters regulation (“Oberflächengewässerverordnung” [45]). Nitrite ($\text{NO}_2\text{-N}$) concentrations up- and downstream of the 3-stage-WWTP were higher than up- and downstream of the 4-stage-WWTP in July (3-stage-WWTP_{upstream} 0.017 mg/L vs. 4-stage-WWTP_{upstream} < 0.003 mg/L; 3-stage-WWTP_{downstream} 0.013 mg/L vs. 4-stage-WWTP_{downstream} < 0.003 mg/L) and August (3-stage-WWTP_{upstream} 0.024 mg/L vs. 4-stage-WWTP_{upstream} 0.003 mg/L; 3-stage-WWTP_{downstream} 0.027 mg/L vs. 4-stage-WWTP_{downstream} < 0.003). In August, higher ammonium ($\text{NH}_4\text{-N}$) concentrations were measured up- and downstream of the 3-stage-WWTP (0.1 mg/L at both sampling sites) than up- and downstream of the 4-stage-WWTP (<0.04 mg/L at both sites). In addition, the orthophosphate concentrations up- and downstream of the 3-stage-WWTP in August were different from those measured before: while the $\text{PO}_4\text{-P}$ concentrations were <0.05 mg/L both up-stream and downstream of the 3-stage-WWTP in July, a month later they reached 0.09 mg/L at the upstream and 0.1 mg/L at the downstream sampling site, which does not correspond to a good ecological status.

Measurements of the water in the exposure tanks at the start and end of both experiments did not show any evidence for a decrease in water quality during the exposure time (for details, see Supplemental Tables S4 and S5).

4. Discussion

In our study, we conducted two experiments with *G. fossarum* sampled at two streams influenced by wastewater of a different quality to obtain information on the heat tolerance

of gammarids. This case study provides important information on different sensitivities of gammarid populations under different exposure scenarios in the field and their capacity to adapt to additional stress in the laboratory. In order to be able to make more general statements, of course, a more extensive study design would be required. However, our study provides the basis for further research to verify our conclusions.

4.1. Effects of Temperature and Life Stage

Both experiments revealed an increasing mortality when gammarids were exposed to heat stress (25 °C). The importance of rising temperatures beyond the physiological optimum for the survivability of organisms, especially gammarids, has been demonstrated in other studies, showing that heat stress is a major factor negatively affecting all physiological and biochemical processes in the organisms [35,36,38,46,47].

Comparing the two experiments, it became evident that, when exposed to elevated temperatures, the mortality of gammarids in the July experiment was much higher than that in the August experiment. The key difference between these two experiments was that we exposed gammarids separated from precopula pairs in July, whereas individually collected gammarids were exposed in August. Due to our study design without triplicates, we can only make the following assumptions based on the results of one aquarium per location/temperature/sex studied. Seasonal variation could also be responsible for the effects we found. However, the differences between the two experiments are so clear that we cannot assume that they are only due to the different sampling times (July vs. August). We have therefore taken a closer look at the differences; in the following, we describe the possible reasons for the effects that we have found. Our results show that gammarids separated from precopula pairs and thus being in the reproductive phase are more sensitive than in the non-reproductive phase. Even at the control temperature, mortality was considerably higher in gammarids separated from precopula pairs than in those individually collected at the sampling sites of the two rivers. This illustrates the enormous stress caused to the animals by the separation from the precopula. The separation of precopula pairs in response to different pollutant exposures can be used as an endpoint in ecotoxicological research with gammarids [48–50]. In some studies, precopula pairs of gammarids are exposed to various environmental toxins; it has been recorded whether pairs are more likely to separate in contaminated environments than under control conditions [48–52]. These studies showed that the formation or separation of precopula pairs can be influenced by various stressors, but the long-term effects of precopula disturbance on individual gammarids have, from our perspective, remained largely unexplored. We are aware that our method to mechanically separate females and males from precopula pairs differs from the behavior of gammarids to terminate precopula by themselves following stress. In the first case, the separation resembles the result of mechanical stress, e.g., posed by heavy rain events. In the second case, the separation is an active response of gammarids to internal stress, which can be used as an endpoint of toxicity. Furthermore, in the context of climate change, the mechanical separation of gammarid precopula pairs may become increasingly important in the natural environment. It is very likely that, following heavy rainfall events, precopula pairs are separated by the extreme hydraulic stress caused by storm-water overflow. As these animals are particularly sensitive during the reproductive phase, it is the more important that, in addition to this hydraulic stress, chemicals also released with untreated wastewater via storm overflow pose stress to them. As reproduction is very energy intensive [53], it is reasonable that, during reproduction, less energy is available for activating cellular defenses, plausibly resulting in the increased mortality rates we have observed in our experiments.

4.2. Differences between Males and Females

Considering the results of the July experiment with males and females separated from the precopula, differences between the sensitivity of females and males became evident. In all cases, the mortality of female gammarids was higher than that of males. Thus, females

appear to be more sensitive than males to exposure-related stress (mortality in the control treatment) and heat stress (mortality in the temperature treatment). This difference in sensitivity between the sexes may also be due to the amount of energy individuals have to allocate while coping with stress: female gammarids have a higher energy requirement in their reproductive phase than males, as oogenesis and egg incubation are more energy consuming than spermatogenesis [54,55]. Furthermore, in the precopula stage, females are on the verge of molting, as the eggs can only be released into the marsupium after molting, where they will be fertilized by the male [56,57]. Thus, in addition to the generally increased energy demand required for oogenesis and egg incubation [54,55], females require additional energy expenditure for the moulting process [58]. Thus, it is physiologically reasonable that they are much more sensitive to stress than males, especially during the reproductive phase. The higher stress sensitivity of female gammarids has also been reported in other studies. Similarly, gammarids have been collected from water bodies and exposed in the laboratory to additional stressors such as cadmium [59], copper [60], and salinity [61]. With the exception of Gismondi, Cossu-Leguille, and Beisel [59], who had sexed individuals on the basis of gnathopod features, in the other studies, female and male gammarids were also determined by separating precopula pairs [60,61], as in the July experiment in our study.

This difference in the sensitivity between females and males, however, was not detectable in animals collected in the non-reproductive phase. Here, we assumed that the sex ratio of randomly exposed gammarids was the same as obtained with a rather large sample size in the field (250 individuals per site). If one sex would have been more sensitive, this would have been reflected by a shifted sex ratio at the end of the experiment. However, the sex ratios determined in the treatments were not significantly different from each other, and no pattern or clear trend was observed at the end of the exposure. We therefore conclude that, in our experiment with gammarids that have not been in the reproductive stage, both sexes responded to stress with equal sensitivity.

Other laboratory studies have already shown that the sensitivity of juvenile gammarids to chemicals was significantly higher than that of adults [62–65]. In our experiment with gammarids that were not in the reproductive stage, we were careful to collect only animals that were exactly the same size as those in the precopula stage. However, a few gammarids were collected in the large field samples (250 gammarids per site), which were later identified in the laboratory as juveniles. Nevertheless, the number of juveniles was vanishingly small (3-stage-WWTP_{upstream} field: 7, 3-stage-WWTP_{downstream} field: 3, 4-stage-WWTP_{upstream} field: 0, 4-stage-WWTP_{upstream} field: 1). In addition, in our exposure experiments with gammarids in the non-reproductive phase, a few individuals were also identified as juveniles at the end of the exposure (3-stage-WWTP_{upstream} 18 °C: 0, 3-stage-WWTP_{downstream} 18 °C: 0, 3-stage-WWTP_{upstream} 25 °C: 0, 3-stage-WWTP_{downstream} 25 °C: 0, 4-stage-WWTP_{upstream} 18 °C: 0, 4-stage-WWTP_{downstream} 18 °C: 1, 4-stage-WWTP_{upstream} 25 °C: 0, 4-stage-WWTP_{downstream} 25 °C: 1). Given the very small number of juveniles that “slipped” into our samples, our results seemed not to be affected by altered juvenile sensitivities.

4.3. Differences between the Two Rivers and Sites—Influence of the WWTPs

When comparing the results obtained for the two rivers in the experiment, with males and females separated from precopula pairs, it became evident that the mortality of both females and males up- and downstream of the 4-stage-WWTP was substantially higher than up- and downstream of the 3-stage-WWTP, both when exposed to the control temperature and to elevated temperature. In male gammarids, this trend was statistically detectable after exposure to 25 °C: the mortality of male gammarids was significantly higher upstream of the 4-stage-WWTP compared to the upstream site of the 3-stage-WWTP, as well as downstream 4-stage-WWTP compared to downstream 3-stage-WWTP. The sampling sites at the 3-stage-WWTP are located about almost 30 km downstream of the source. Several point sources discharge into the river upstream of our sampling sites, including the WWTP Metzingen (120,000 population equivalents) and more than 30 storm-

water overflow basins (see Figure 1). This presumably leads to a high micropollutant load upstream of our sampling site upstream of the 3-stage-WWTP, which is also reflected by a moderate ecological status of the river (results of the 2015 Water Framework Directive River Basins Management Plan). However, it has also long been known that wastewater treatment plants have a significant impact on the composition and quantity of nutrients in the receiving waters and thus contribute significantly to increasing nutrient loads [66,67]. As a result of the WWTP Metzingen, nutrient loads at sampling sites up- and downstream of the 3-stage-WWTP are likely to be much higher than that of the sampling sites up- and downstream of the 4-stage-WWTP, where no other treatment plant is located upstream. In addition, the sampling site upstream of the 4-stage-WWTP is located just about 8 km downstream of the source, with only 10 storm-water overflow basins discharging into this stream section (see Figure 1). In addition to the lower load of micropollutants, the nutrient content in the river Eyach (connected to the 4-stage-WWTP) is also lower than in the Erms (connected to the 3-stage-WWTP), as indicated by our limnological data (see Section 3.3). The comparison of the two WWTPs (3-stage-WWTP vs. 4-stage-WWTP) also showed differences in the nutrient content of the respective effluents. The annual average of the chemical oxygen demand (COD) in the effluent of the 3-stage-WWTP was 15.8 mg/L compared to 6.0 mg/L in the effluent of the 4-stage-WWTP. Considerable differences were also observed with respect to the concentrations of ammonium ($\text{NH}_4\text{-N}$, annual average) in the effluents: A concentration of 0.3 mg/L was measured in the effluent of the 3-stage-WWTP, while the effluent of the 4-stage-WWTP contained only 0.03 mg/L (see Supplemental Tables S1 and S2). Thus, in the Erms river (connected to the 3-stage-WWTP), gammarids appear to have more nutrients available to meet their increased energy needs than in the river Eyach (connected to the 4-stage-WWTP), making them less susceptible to stress caused by separation from precopula pairs. This is reflected in the lower mortality of male and female gammarids in the Erms (3-stage-WWTP) compared to the Eyach (4-stage-WWTP), regardless of whether the sampling sites are upstream or downstream of the WWTP.

In addition to differences in the thermotolerance of gammarid populations between the two rivers, differences could also be observed between the upstream and downstream sites in respect to WWTPs. Upstream and downstream of the 3-stage-WWTP, gammarids showed similar tolerance to the temperature increase, with no differences in mortality between the two sampling sites. In contrast, the gammarids reacted differently upstream and downstream of the 4-stage-WWTP, which is equipped with a powdered activated carbon stage. The mortality following heat stress was significantly lower downstream of the 4-stage-WWTP compared to the upstream site (mortality of 30% downstream 4-stage-WWTP vs. 54% upstream 4-stage-WWTP). As mentioned above, several point sources already discharge into the Erms (connected to the 3-stage-WWTP) upstream of the sampling sites, including the WWTP Metzingen and many storm-water overflow basins. This presumably leads to a high micropollutant load in the Erms upstream of the sampling site 3-stage-WWTP_{upstream}. The conventional 3-stage-WWTP may be likely to further release micropollutants into the river at the sampling site downstream of the 3-stage-WWTP, but it may be questionable whether this additional input, together with a high volume of purified water, substantially increases the micropollutant concentrations downstream of the 3-stage-WWTP. The sampling sites connected to the 4-stage-WWTP, on the other hand, are located about just 8 km downstream of the source. Even though this river in its upstream part may be impacted by micropollutants from diffuse sources, the 4-stage-WWTP is the first WWTP to discharge into the river, with only 10 storm-water overflow basins upstream, resulting in a better ecological condition compared to the Erms river (connected to the 3-stage-WWTP). For more than 30 years, the 4-stage-WWTP has been equipped with a powdered activated carbon stage, which effectively reduces the discharge of micropollutants [31,32,68]. So, the very effectively purified effluent released by the comparatively large 4-stage-WWTP may dilute existing pollution that has resulted from stormwater overflows or agricultural run-offs. If this interpretation holds true, gammarids sampled downstream the 4-stage-WWTP

would be exposed to lower concentrations of contaminants and thus require less energy to detoxify contaminant loads, which can then be allocated to necessary biochemical and physiological responses to heat stress, resulting in an overall lower mortality downstream of the 4-stage-WWTP. Many studies have already shown that the health of animals improves significantly when a fourth treatment stage is added to WWTPs [29,32–34,69], as it is the case also in a river adjacent to the Eyach with a comparable equipment of the WWTP for more than 30 years [70]. Thus, it seems very plausible that in the present study, gammarids sampled at the Eyach downstream of the 4-stage-WWTP have profited from this technology by increasing their tolerance to temperature rise.

An integrative view on the situation in the two investigated streams that explains the entirety of our data, however, requires the proposal for microevolutionary effects that have led to the selection of unspecifically tolerant phenotypes in the river Erms connected to the 3-stage-WWTP. The selection for tolerance in amphipods has already been shown by Anguiano et al. [71] for *Hyalella curvispina*, and has also been reported for other freshwater crustaceans [72,73]. An interaction of freshwater pollution and the origin of gammarid populations defining the stress response of transplanted *G. fossarum* individuals to their habitat has been found by Schill and Köhler [74]. According to the view proposed here, the long-lasting (limno-)chemical situation in the rather polluted river Erms (connected to the 3-stage WWTP) has led to a selection of phenotypes that show a higher tolerance to physiological challenges (high nutrient burden; micropollutants) in the reproductive phase, possibly based on an increased metabolic rate or a more effective energy supply than in wild-type strains. This assumption would also have led to a certain ‘cross-tolerance’ of the Erms (3-stage-WWTP) animals to increased temperature in the reproductive phase: elevated temperature may be still disadvantageous for the Erms (3-stage-WWTP) population, but not as tragic as for the animals from the Eyach river (connected to the 4-stage-WWTP) that did not undergo a selection for tolerance. In contrast to the river Erms (3-stage-WWTP), wild-type phenotypes in the less polluted river Eyach (4-stage-WWTP) seem to have persisted following the lack of selection pressure by chemicals. For such a population, however, our results indicate that severe stress situations during the reproductive phase may be fatal due to lacking ‘cross-tolerance’. Therefore, in selected cases, the advantage of a fourth purification step to reduce chemical pollution seems to trade off against pollution-induced ‘cross-tolerance’ to other strong stressors as, e.g., mechanical disturbance combined with elevated temperatures. It is particularly necessary here that the ecotoxicological situation downstream the 4-stage-WWTP remains as good as possible, because additional mechanical stress cannot be tolerated by the organisms. In order to allow wild-type phenotypes to allocate energy reserves to thermotolerance the best way possible and thus to tolerate elevated temperatures at least in the non-reproduction phase, expanding wastewater treatment plants with additional purification stages is an absolutely appropriate method.

4.4. Relevance of Our Results in the Context of Climate Change

In the context of climate change, our results are of great relevance. In some regions of Austria and Germany, summer precipitation is predicted to decrease by about 40% by 2100, which could increase periods of low dilution potentials for wastewater in surface waters [75]. Models by Abily et al. [76] show that climate change impacts will lead to a consistent 11% decrease in the dilution factor for introduced chemicals. By consequence, this will increase the concentration of micropollutants, especially downstream of WWTPs that are not equipped with a fourth purification stage. Abily et al. [76] have estimated that up to 42% of the streams receiving WWTP discharges and currently reaching a good ecological status show a 70% probability to have their ecological status downgraded due to climate change. In this context, it is more important to reduce the discharge of micropollutants via WWTPs in order to keep the basic stress level of wild-type populations in the receiving streams as low as possible. This will allow those gammarids that have not undergone selection—and this is the vast majority of phenotypes in streams with a good

ecological status—to develop a higher resilience in the face of co-exposure to additional and concomitant climate change-related stressors, as our experiments have shown.

Obviously, our assumptions can only be made on the basis of single aquaria, two selected wastewater treatment plants and two different rivers. We recognize that other factors may have influenced the effects we found. As mentioned at the beginning of the discussion, a more extensive study design would be required in order to be able to make statements that are more general. However, our case study provides the basis for further research to verify our conclusions. So far, our results show that the sole expansion of individual WWTPs with a fourth purification stage does not appear to be sufficient to increase the tolerance, particularly of female *G. fossarum*, to extreme temperatures plus mechanical stress in the sensitive phase of reproduction. As climate change is expected to increase water temperatures during the summer months when these gammarids reproduce, it is also imperative to reduce inputs to the upper reaches of watercourses that are not directly related to WWTP discharges, either through storm overflows or diffuse sources, in order to ensure the persistence of gammarid populations under climate change conditions. The current disappearance of formerly large gammarid populations in many small and medium-sized watercourses [77] may well be due to the action of micropollutants on them, as shown in our study.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pollutants4010004/s1>, Table S1: Discharge characteristics of the wastewater treatment plant (WWTP) Bempflingen-Riederich in the year 2020; Table S2: Discharge characteristics of the wastewater treatment plant (WWTP) Albstadt-Lautlingen in the year 2020; Table S3: Limnological parameters measured during gammarid sampling in July (sampling of precopula pairs) and August (sampling of gammarids in the non-reproductive phase) 2020; Table S4: Limnological parameters at the end of exposure of gammarids separated from precopula pairs at 15 °C and 25 °C. Exposure duration: 13.07.–20.07.2020; Table S5: Limnological parameters at the end of exposure of gammarids in the non-reproductive phase at 18 °C and 25 °C. Exposure duration: 04.08.–11.08.2020; Table S6: Exposure of male and female gammarids separated from precopula pairs; Table S7: Exposure of gammarids in the non-reproductive phase.

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