

Size-dependent sensitivity of stream amphipods indicates population-level responses to chemical pollution

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Abstract

1. Global change assessments have typically ignored synthetic chemical pollution, despite the rapid increase of pharmaceuticals, pesticides, and industrial chemicals in the environment. The paucity of research on the ecological effects of these *micropollutants* undermines our efforts to address the freshwater biodiversity crisis. Understanding the responses of individual organisms to chemical pollution should help address this knowledge gap because individual-level effects can cascade across populations, communities, and ecosystems with devastating consequences.
2. Inputs of treated municipal wastewater are a major source of micropollutants in receiving environments. Here, we assessed population and individual-level influences of treated wastewater on freshwater gammarid amphipods (*Gammarus* spp.) in Swiss lowland streams in situ and tested effects of a micropollutant mixture on individual *Gammarus fossarum* using a common garden laboratory experiment. We hypothesised that population-level effects of wastewater are mediated through asymmetric sensitivities of juvenile and adult gammarids to chemical pollutants. We expected that life-stage specific sensitivities would reflect allometric theory relating body size to a wide range of organismal characteristics, including metabolism, growth, and mortality.
3. At the population level (i.e. field survey), we observed greatly reduced abundances of juvenile gammarids downstream of the wastewater discharge in three of the six sites surveyed, indicating the potential for demographic effects of pollution. At the individual level in a field transplant experiment, we found that the presence of wastewater led to a steeper positive relationship between gammarid body size and leaf consumption. In the laboratory experiment, we found that micropollutants had negative effects on consumption and growth rates, but a positive effect on survival. Differences in the relationship between gammarid body size and performance were subtle across treatments, although flatter slopes in growth appear to be a consistent response to chemical pollution. Faster growth rates appeared to be connected with reduced survival, placing individuals in control treatments and/or with smaller body size at greatest risk of mortality. Notably, juvenile

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gammarids had faster growth rates and lower survival than adults when exposed to micropollutants.

4. Our results demonstrate the potential for negative impacts of micropollutants in freshwater ecosystems. However, the results also show that organismal responses to chemical pollutants can be complex, whereby impacts at the individual level may act counter-intuitively to population-level dynamics. This highlights the need for more realistic experiments to better assess how organismal responses depend on life stage and body size, and how individual-level effects propagate to higher levels of biological organisation. Our study shows how allometric theory can be used to examine the effects of stressors on underlying organismal biology, population demographics, and link with broader macroecological patterns.

KEYWORDS

body size, Gammaridae, Micropollutants, multiple stressors, wastewater

1 | INTRODUCTION

Humans are drastically altering the Earth's ecosystems through multiple drivers, including climate change, landscape disturbance, and chemical pollution (Tylianakis, Didham, Bascompte, & Wardle, et al., 2008). As a consequence, global biodiversity is changing at an unprecedented rate (Tilman et al., 2017), with freshwaters disproportionately affected (Harrison et al., 2018; Reid et al., 2019). In particular, human land uses, water extraction, and eutrophication lead to losses of biodiversity, altered community composition, and reduced ecosystem functioning (Burdon et al., 2013, 2020; Woodward, 2009). However, the effects of *emerging* contaminants (e.g. synthetic chemicals and plastics) on aquatic ecosystems remain less well understood (Bernhardt et al., 2017; Windsor et al., 2019).

Environmental stress, such as chemical pollution, influences species distribution, abundance, and biotic interactions in a wide range of ecosystems (Forbes, 1999). Interspecific variation in sensitivity to toxicants is well-established and can reflect variation in species traits related to toxicokinetics (uptake, distribution, biotransformation, elimination) or toxicodynamics (interaction with biological target sites; Nyman et al., 2014). Importantly, both inter- and intraspecific variation in responses to stressors may be strongly tied to body size. Allometry, in its broadest sense, describes how the characteristics of organisms change with size (Shingleton, 2010), and has been at the forefront of advances in macroecological theory, including the metabolic theory of ecology (MTE; Brown et al., 2004). Allometric scaling laws relate body size to a wide range of organismal characteristics including metabolism, growth, abundance, and mortality (Hatton et al., 2019). Consequently, stressor effects may strongly vary between species, phenotypes, or life-history stages due to differences in body size (Adam et al., 2010; Alonso et al., 2010; Moore & Folt, 1993). For instance, early life stages of insects, crustaceans, and fish are frequently more sensitive to toxicants than larger, more mature stages (e.g. Alonso et al., 2010; Cadmus et al., 2020;

Hutchinson et al., 1998), possibly due to size-based processes described by allometric theory (Townsend & Thompson, 2007). These theoretical expectations and empirical observations underpin the importance of investigating how intraspecific and ontogenetic variation in body size influence population demographics and responses to environmental stress, including chemical pollutants (Forbes & Depledge, 1996).

Pollution from synthetic chemicals is a pervasive threat to biodiversity and ecosystem services (Bernhardt et al., 2017), and *micropollutant* (MP) contamination can affect freshwater ecosystems at local to continental scales (Malaj et al., 2014; Stamm et al., 2016). Micropollutants typically comprise a wide array of substances including pharmaceuticals, personal care products, industrial chemicals, and pesticides present at very low concentrations (from ng/L to µg/L; Schwarzenbach et al., 2006). Micropollutants can enter aquatic environments in runoff from farmland (Pedersen et al., 2005) and roads (Vystavna et al., 2013), but normally the greatest source is treated municipal wastewater (WW) discharges (Luo et al., 2014). Exposure to MPs may increase environmental stress at different levels of biological organisation: from the cellular (Ren et al., 2009), through the individual (Bundschuh et al., 2011) and community (Munz et al., 2016), to the ecosystem (Halstead et al., 2014).

However, detecting the biological effects of MPs in an ecologically relevant context is challenging (Stamm et al., 2016). Firstly, effects of chemicals studied in the laboratory may not accurately predict responses in real ecosystems, where MPs with different modes of action often co-occur in varying concentrations (Fischer et al., 2013). Furthermore, MPs may interact with other chemical and non-chemical stressors (Schäfer et al., 2016), further adding to the biological complexity generated through trophic and non-trophic pathways (Halstead et al., 2014). Interactions among multiple stressors can cause non-additive effects, potentially acting synergistically or antagonistically in combination (Jackson et al., 2016). Secondly, many studies have focused on acute lethal toxicity of different

chemicals (Kunz et al., 2010), whereas sub-lethal effects of chronic exposure are less well understood (Nyman et al., 2014). Yet, both lethal and sub-lethal effects of environmental stressors (e.g. MPs) can directly and indirectly affect individual fitness and population structure (Calow & Sibly, 1990), with consequences for higher levels of biological organisation (Desneux et al., 2007; Galic et al., 2018).

Here, we tested individual and population-level effects of pollution using freshwater gammarid amphipods (*Gammarus* spp.) as a model system. We focused on urban streams, where discharges from municipal WW treatment plants (WWTPs) are a major source of chemical pollution (Burdon et al., 2016). Wastewater is known to affect gammarid populations (Gross et al., 2001; Ladewig et al., 2005), with MP toxicity hypothesized as a dominant stress pathway (Bundschuh et al., 2011). To increase our inferential power about individual and population-level effects of chemical pollution, we first conducted a field survey. We used the survey to compare the abundances and size-structure of gammarid amphipod populations between impacted (below WWTPs) and unimpacted reference (above WWTPs) locations in multiple streams. Next, we performed a field transplant experiment to test how WWTP discharge affects individual performance of amphipods (juvenile gammarids and adult *Gammarus pulex* and *Gammarus fossarum*). Finally, we conducted a laboratory experiment to study individual-level effects of a realistic mixture of MPs on *G. fossarum*.

We made three main predictions. Firstly, if pollution from WWTPs has asymmetric effects on different life stages (i.e. juveniles and adults, Figure 1a,b), then population sizes and size structure should differ between sites upstream and downstream of WWTPs (field survey). Secondly, if life stage sensitivity to chemical pollution is asymmetric (i.e. Figure 1c,d), then individual adults and juvenile gammarids should differ in performance when exposed to treated effluent (field experiment) and MPs (laboratory experiment). Thirdly, we predicted that responses would scale with individual body size (i.e. due to allometric processes), but the slopes of these relationships would differ with exposure to chemical pollution reflecting size-dependent responses to environmental stress. For example, if juvenile gammarids are more sensitive to chemical pollution (e.g. reduced leaf consumption), then we predicted a steeper slope in the body size-response relationship (Figure 1e). Alternatively, greater adult sensitivity could lead to a flatter slope of the body size-response relationship (Figure 1f), or vice versa (depending on the responses tested: consumption, growth, and survival). For survival, we hypothesised that there should be a slope of 0 if mortality is invariant (i.e. equivalent) with body size (assuming a binary response where 0 = dead and 1 = alive). However, depending on the asymmetry of the response, the slope could be negative if there is an adult-bias in mortality, or positive if juveniles are more sensitive. Finally, we considered that responses in the field and laboratory assays could be idiosyncratic due to biological and/or environmental contingencies that can result in *ecological surprises* (Jackson et al., 2016). Evaluating these predictions would help us better understand the mechanisms underpinning organismal responses to chemical pollution in freshwater ecosystems.

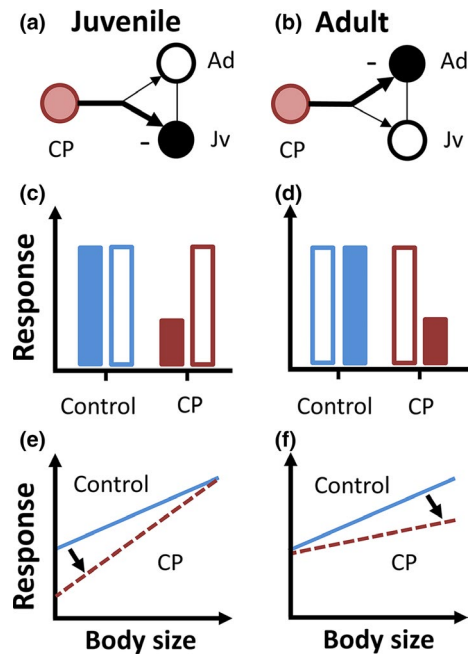


FIGURE 1 Our main hypotheses focused on asymmetric sensitivities of gammarid amphipod juveniles (Jv; a, c—solid bars, e) and adults (Ad; b, d—solid bars, f) to chemical pollution (CP). We hypothesised that (c) juvenile or (d) adult gammarids (after accounting for individual body size) might indicate asymmetric sensitivities to CP relative to reference conditions (Control) using indicators of performance (e.g. resource acquisition). Considering body size explicitly, greater sensitivities of juveniles could lead to a steeper slope in the size-response relationship (e), or greater sensitivities of adults could lead to a flatter slope in the body size-response relationship (f). Chemical pollution includes wastewater (WW; Field) and micropollutants (MP; Laboratory). The solid line between adults and juveniles in the field populations (a, b) indicates the linkage between the two gammarid life stages in the field populations; this linkage was not explicitly considered in field and laboratory experiments because only individual responses were tested [Colour figure can be viewed at wileyonlinelibrary.com]

2 | METHODS

2.1 | Study organism

Gammarid amphipods are functionally important *keystone* consumers in the Northern Hemisphere, often dominating benthic macroinvertebrate community abundances and biomass (MacNeil et al., 1997; Woodward et al., 2008). They are opportunistic, omnivorous scavengers that contribute to detrital processing (*shredding*) in stream ecosystems, are intermediate hosts for a range of parasites, and important prey for fish and birds (MacNeil et al., 1999; Westram et al., 2011). Their sensitivity to pollutants make them valuable indicators of ecosystem health and a useful model system for aquatic ecotoxicological studies (Kunz et al., 2010). In Switzerland, at least eight different species of *Gammarus* (Amphipoda: Gammaridae) occur (Altermatt et al., 2014), and both *G. pulex* Linnaeus, 1758 and *G. fossarum* Koch, 1835 were abundant at our study sites. Our field survey and transplant experiment included both species, but the

laboratory experiment used only *G. fossarum*. For further information, see Appendix S1.

2.2 | Study sites

We conducted field surveys at six WW-impacted streams in Switzerland over two consecutive years (2013 and 2014; see Appendix S1: Table A1 for more details). Three study sites were in the Jura Mountains (Val-de-Ruz, Villeret, Niederdorf) and three on the Swiss Plateau (Knouau, Kernenried, Hochdorf). Sampling locations were located upstream (US) and downstream (DS) of the WWTP discharge, with the DS site located where WW was completely mixed at base flow conditions (Burdon et al., 2016). The in situ transplant experiment using adult *G. pulex*, adult *G. fossarum*, and gammarid juveniles was performed at the site Knouau. For the laboratory experiment, we collected *G. fossarum* individuals from an unimpacted stream (Bäntalbach). More details regarding the study design and sites are provided in Appendix S1, including a site map (Figure A1).

2.3 | Field survey

We used quantitative benthic sampling to investigate the taxonomic composition, densities, life stage structure, and other demographic features of gammarid populations at both US and DS sites in each of the six study streams. Eight replicate Hess samples (mesh size 250 µm, area 0.04 m²) per sampling location (US, DS) were taken to collect gammarids from run/riffle habitat in the thalweg of each of the six streams. Each benthic sample was preserved in 90% ethanol and stored for processing. In the laboratory, gammarids were separated into juveniles (<7 mm) and adults (≥7 mm) following Adam et al. (2010). Our own investigations of female gammarid reproduction suggest that the ≥7 mm threshold is valid, with pregnant females measuring an average length of 11.32 mm (10.81–11.84 mm, 95% CI). Juveniles were not identified to species because it is difficult to discern the morphological features (i.e. the length of the outer and inner ramus on uropod 3) used to differentiate *G. fossarum* from *G. pulex* (Altermatt et al., 2019) when individuals are small. We observed a large number of intersex individuals during the field survey; intersex individuals are those that possess female attributes (e.g. four pairs of oostegites) without the loss of male attributes (e.g. one or two penial papillae, enlarged posterior gnathopods) as recorded in the literature (see Ford et al., 2003; Ladewig et al., 2003; Short et al., 2014).

We assessed gammarid population body-size (age) structures from the Hess samples collected at US and DS locations in the three sites (Niederdorf, Hochdorf, Kernenried) sampled in 2013. Individual size was measured from digital images (see Section 2.4). We measured all adult individuals, but due to large numbers of juveniles, a subset of 20 individuals were measured per sample and assigned to size classes ranging from ≥2.5 to <7 mm in increments of 0.5 mm.

The relative size-class abundances of these 20 individuals were then used to estimate the size distributions of all juveniles per sample. To assess fecundity, we collected 93 gravid females using kick-net sampling at US/DS locations in three 2014 sites (Appendix S1: Table B1). For more details on the field survey, see Appendix S1.

2.4 | Size measurements

We recorded gammarid body size (to the nearest 0.001 mm) from digital images using the ImageJ software (1.48v). Images of individual gammarids placed sideways were taken using a Canon EOS 60D (Canon lens EFS18–135 mm, macro setting), with a mm paper background for scale estimation. Measurement followed the curved outline of the amphipod from the base of the first antennae to the base of the third pair of uropods (Danger et al., 2013). Measuring amphipod curvature digitally is more accurate and precise than straightening (Quigley & Lang, 1989) and can be achieved without harm to the animal. The measurement error (RSD, relative standard deviation) was 3.52% for adults and 3.62% for juveniles; these were estimated from size measurements of 14 individuals (seven adults and seven juveniles), each measured 10 times (i.e. from 10 digital images of the same live individual reset for each photograph). See Appendix S1 for more details.

2.5 | Leaf consumption

The consumption of leaf discs by individual gammarids was used as an indicator of stress in both experiments (Maltby, 1992). We prepared standardised leaf discs (Ø 1.5 cm) from abscised and air-dried alder (*Alnus glutinosa*) leaves. Leaf discs were pre-conditioned at 16°C in oxygenated aquaria filled with stream water for 8 days. We measured leaf-disc area before and after each experimental period using ImageJ software (1.48v). Leaf discs were digitally scanned (JPEG format, 300 dpi, Xerox Workcentre 7855) and each image included a mm ruler for scale estimation. The measurement error was 0.13% (Appendix S1: Table B2). Consumption was defined as % change in leaf area per day using the following equation:

$$\text{Consumption} = \frac{\frac{A_i - A_f}{A_i} \times 100}{t} \quad (1)$$

where A_f is the final leaf disc area, A_i denotes the initial leaf disc area, and t the time (days) elapsed.

2.6 | Field transplant experiment

The field experiment tested the performance (consumption, growth, and survival) of transplanted upstream gammarids in response to diluted WW at our study site Knouau in late autumn 2014. We collected gammarids upstream from the WW input, visually screened

them for parasites under a stereo microscope, and sorted individuals into three groups (juveniles, adult *G. fossarum* and adult *G. pulex*) before photographing to enable initial size estimation (see Section 2.4). We placed five cage complexes (consisting of 10 cages in arrays) in similar mesohabitats above and below the WW input. Cages were oriented perpendicular to flow direction at mid-water depth, following a method similar to the *GamTox* assay (Gerhardt, 2011). Each cage had two pre-conditioned alder leaf discs (see Section 2.5). We randomly allocated one control (no amphipod), three adult *G. pulex*, three adult *G. fossarum*, and three juveniles to each cage complex leading to 15 replicates per gammarid treatment. The experiment lasted 11 days, after which surviving individuals were enumerated and photographed to enable growth estimations. We used length measurements from digital images to calculate amphipod relative growth rates (Glazier et al., 2011). The relative growth rate represents the proportional daily growth increment (Eq. 2):

$$\text{Relative growth rate} = \frac{\ln(L_F) - \ln(L_I)}{t} \quad (2)$$

where L_I is the initial length, and L_F denotes the final length at the end of the experiment, and t the time (days) elapsed.

In the field transplant experiment, temperatures differed between locations, with the DS location being warmer by a mean 0.495°C per day over the study period. Consistent with the MTE (Brown et al., 2004) and a meta-analysis of feeding experiments (Rall et al., 2012), our response variables (R), feeding and growth rates, are expected to depend on both temperature and body size. Following Gillooly et al. (2001), we approximated R as the combined effects of body size and temperature (within a biologically relevant temperature range) using Eq. (3):

$$R \sim M^{b_R} e^{-E_R / kT} \quad (3)$$

Kratina et al. (2019) applied these theoretical expectations in Eq. (4):

$$R = R_0 M^{b_R} e^{\frac{E_R}{k} \frac{T_R - T_0}{T_R T_0}} \quad (4)$$

where R_0 is the feeding or growth rate at T_0 , M is body size (e.g. mass, but here we used length [mm]), b_R is an allometric exponent, E_R is the activation energy of the biochemical reactions underpinning R (eV), k is the Boltzmann constant (8.6×10^{-5} eV/K), T_R is the location temperature (K), and T_0 is the standard temperature (here $T_0 = 10.478^\circ\text{C} = 283.63$ K; the midpoint of the temperature range between locations). We performed multiple linear regression on the natural logarithm of Eq. 4 to assess the main effects of temperature and body size on R (Kratina et al., 2019). We then divided feeding and growth rates by the universal temperature dependence $e^{E_R T / k T_R T_0}$ (Gillooly et al., 2001), where $T = T_R - T_0$ (see also Eq. 4). This correction meant we could assess the influence of body size on R after accounting for the warming effect associated with the WW input. For more details on the field transplant experiment, see Appendix S1.

2.7 | Laboratory experiment

We tested performance (consumption, growth, and survival) of *G. fossarum* in response to MPs using a laboratory experiment with a 2×4 factorial design. Individual gammarids from two life stages (Juvenile, Adult) were exposed to four different water treatments (Control, Technical Control, Low, and High MP concentrations) for 21 days under semi-static conditions (Appendix S1: Table B3). The technical control accounted for the carrier (methanol, MeOH) used to keep the MPs in solution for dosing; laboratory-grade MeOH only was dosed at an equivalent concentration to that in High MP treatment (approximately 5.76 mg/L). The MP mixture (Table 1) consisted of 18 different chemicals representing different compound classes with specific modes of actions that typically occur in Swiss WW (Burdon, Bai, et al., 2020; Stamm et al., 2016). One of the compounds used was the pesticide diazinon, a known toxicant to gammarid amphipods (Mayer & Ellersieck, 1986; Nyman et al., 2014). Further, following the methods described in Munz et al. (2017), we calculated the expected toxic units (TUs) for the Low MP treatment as 0.070 TUs and 0.525 TUs for the High MP treatment (or -2.66 and -0.64 on the log scale, respectively). These estimates excluded oestradiol and metformin used in the mixtures because, to the best of our knowledge, ecotoxicological information for these compounds is lacking. For further details on the MP treatments including realised concentrations in each treatment (Appendix S1: Table B4).

We used three shelving Units with three shelves (Level) in a temperature-controlled environment (16°C). Nine trays (Tray), each containing 12 glass microcosms (Bauer, Joghurtglas 250 g; h = 104 mm, $\varnothing = 70$ mm, V = 284 ml), were placed on each level. In March 2015, we collected adult and juvenile amphipods of the *G. fossarum* species complex from a relatively pristine forested stream (Bäntalbach) in Canton Zürich, Switzerland and acclimated them to laboratory conditions. Forty adults and 27 juveniles were allocated to each treatment; each adult was provided two pre-conditioned alder leaf discs, juveniles one. We stratified-randomly allocated treatment combinations to every tray. Microcosms were aerated through PVC tubing (0.5 cm \varnothing) entering a glass pasteur pipette and covered with parafilm to reduce evaporative losses. We exchanged water and leaf discs weekly and monitored survival daily. We used image analyses to estimate growth (see Section 2.4) and consumption (see Section 2.5). We collected water samples to measure MP concentrations (Appendix S1: Table B4) and describe the general water chemistry (Appendix S1: Table B5) of each treatment. For more method details, see Appendix S1.

2.8 | Data analysis

2.8.1 | Field survey

We tested the presence of WW on gammarid abundance, size (age) structure, species composition, adult body size, and sex ratios. We used a generalised linear mixed-effect model (GLMM) assuming a

Group	Description	Substance	Low MP	High MP
			ng/L	ng/L
Food additive	Sweetener	Sucralose	156	1,171
Heavy metal	Metal alloy	Zinc	800	6,000
Industrial chemical	Corrosion inhibitor	Benzotriazol	146	1,095
Pesticide	Insecticide	Diazinon	60	450
	Herbicide	Diuron	10	74
	Fungicide	Tebuconazol	3	24
PCP	Antimicrobial	Triclosan	7	55
Pharmaceutical	Antipsychotic	Amisulprid	14	105
	Beta-blocker	Atenolol	29	215
	Antihypertensive	Candesartan	24	180
	Anticonvulsant	Carbamazepin	37	280
	Antidepressant	Citalopram	7	55
	Antibiotic	Clarithromycin	8	63
	Analgesic	Diclofenac	80	600
	Oestrogen	Oestradiol	0.05	0.35
	Antihistamine	Fexofenadin	43	320
	Contrast media	Iopromid	217	1625
	Antidiabetic	Metformin	667	5,000

TABLE 1 Micropollutants (MP) used in the laboratory experiment testing individual responses of *Gammarus fossarum*. Two concentrations of a MP mixture (Low, High) were used to simulate realistic scenarios in Swiss streams receiving inputs of treated municipal wastewater (Burdon, Bai, et al., 2020; Stamm et al., 2016). PCP, personal care product

Poisson distribution to analyse gammarid abundances, where Hess sample *Replicate* nested in *Location* and *Site* was a random effect, and *Year* (2013, 2014), sampling *Location* (US, DS), gammarid *Life stage* (Juveniles, Adults), and the interaction of location and life stage the fixed effects. *Year* was dropped from the final model after likelihood ratio testing indicated that it was redundant. Adult species composition (proportions of *G. fossarum* and *G. pulex*) from Hess samples was tested using a generalised linear model (GLM) assuming a binomial distribution with the predictors sampling location, site, and year. The Val-de-Ruz site has an upstream subsurface connection to another WWTP that was undetected during site selection (Burdon et al., 2019), so we excluded this site from our analyses unless otherwise specified.

Invertebrate body size can be influenced by chemical pollution (Moore & Folt, 1993), and we hypothesized that individuals from both species (*G. fossarum* and *G. pulex*) would be smaller downstream of the WWTPs. We tested for differences in adult size (mm) from the 2013 Hess samples using a GLM where the predictors were sampling location, gammarid species (*G. pulex*, *G. fossarum*), site, and the interaction of location and species. Size was log-transformed to improve normality and reduce heteroscedasticity. Sex ratios in gammarids may be influenced by chemical pollution (Ford et al., 2006), and we hypothesized that there could be more intersex individuals downstream of the WWTPs. We tested for shifts in the ratio of female and intersex individuals from the 2013 samples using a GLM assuming a binomial distribution with predictors sampling location, species, site, and the interaction of species and location. Indeterminate and male individuals were not analysed (males were observed infrequently at

generally very low abundances). Reduced fecundity can limit population growth (Moore & Folt, 1993), and we hypothesized that female fecundity could be lower at locations below WW inputs. We analysed female fecundity from the 2014 sites (Knonau, Val-de-Ruz, Villeret) as the number of eggs per individual using a GLM assuming a Poisson distribution for count data with predictors sampling location, species, site, and the interaction of species and location. Female body size and its influence on fecundity were tested in separate GLM.

2.8.2 | Field transplant experiment

We used temperature-corrected consumption and growth as response variables *R* to test species, life stage, and size-dependent sensitivities to the presence of WW in the field transplant experiment. Gammarid leaf consumption (Eq.1) and growth (Eq.2) were analysed using linear mixed-effects (LME) models, reduced major axis (RMA) regressions, and effect size analyses. Responses were considered for surviving individuals only (94% of all gammarids used). Consumption (expressed as the % change in leaf area per day) was $\log[x + 1]$ -transformed after corrections for leaf expansion (see Appendix S1 for more details). Growth (change in log-transformed length [mm] per day) was untransformed because it had a normal distribution with positive and negative values (i.e. shrinkage in length). Both responses were corrected for temperature influences (see Section 2.6). In the LME models, we first tested the exposure *Location* (US, DS), gammarid *Type* (juveniles, adult *G. fossarum* and *G. pulex*) and

their interaction as fixed effects with the cage *Complex* as a random effect. Secondly, we tested the same LME model with standardised individual log-transformed body size (mm) as a covariate. We include both models in the results. Thirdly, we explicitly tested the interaction between standardised individual body size and exposure location (i.e. how the size-scaled response changed with location) using LME models with gammarid type as fixed control variables, and cage complex as a random effect. Log-transformed body size (mm) was standardised (centred on the mean and scaled to unit variance) to help provide more meaningful slope comparisons across treatments (Schielzeth, 2010). Survival responses were not analysed as only five individuals died during the experiment and there was no obvious pattern in mortality.

An important issue for line-fitting in allometry is that typically, two sources of error are present (i.e. measurement and equation error), which have different implications for the choice of statistical methods (Warton et al., 2006). Unaccounted measurement error in the predictor variable can lead to divergent slope estimates (Green, 1999). Thus, we used a model II regression method, RMA regression, in addition to our LME models. Reduced major axis provides a valuable alternative to least-squares regression approaches and is commonly used in allometric studies (Green, 1999; Warton et al., 2006). To fit RMA regression models, we used the *lmodel2* R package using log-transformed body size (mm) as the predictor variable.

2.8.3 | Laboratory experiment

To test life stage and size-dependent sensitivities to the presence of MPs in the laboratory, we used consumption (Eq. 1), growth (Eq. 2), and survival as response variables in mixed models, RMA regressions, and effect size analyses. Consumption (% change in leaf area per day) was $\log[x + 1]$ -transformed after corrections for leaf expansion (Appendix S1). Growth (change in log-transformed length [mm] per day) was untransformed because it was normally distributed with positive and negative values (i.e. body length shrinkage).

Firstly, we tested consumption by surviving gammarids (i.e. excluding dead individuals) with the fixed effects *Life stage* (Juvenile, Adult), *Treatment* (Control; Technical control, MeOH; and Low and High MPs) and their interaction term. Standardised initial log-transformed body size (mm) was included as a covariate in an alternative model; we include both models in our results. These models included random effects with *Individuals* and *Week* to account for repeated measures and *Tray* nested in *Level* and *Unit* for experimental blocking. We then explicitly tested the interaction between standardised initial log-transformed body size and treatment, including life stage as a fixed covariate and the same random effects as specified for the first two consumption models. Life stage was included as a fixed effect in all consumption models because juveniles and adult gammarids had different feeding regimes (see Section 2.7).

We tested growth using an LME model with surviving individuals only. The fixed effects included life stage and treatment, and the

random effect tray nested in level and unit for experimental blocking. Standardised initial log-transformed body size was included as a covariate in an alternative model; both models are included in the results. We then explicitly tested the interaction between standardised initial log-transformed body size and treatment, with the same random effects as specified for the first two growth models.

We first analysed gammarid survival at the end of the experiment (21 days) using a GLMM with a binomial distribution. The fixed effects were life stage, treatment, and their interaction; the random effect was tray nested in level and unit for experimental blocking. We fitted the same model with standardised initial log-transformed body size as a covariate. We report results from both models in the results (see Appendix S1: Table C13). We also dropped the interaction term from the first model to test the main effects of Life stage and Treatment. Thirdly, we explicitly tested the interaction between standardised initial log-transformed body size and treatment on survival. The same random effects were used as specified for the first two survival models.

We fitted LME and GLMM models using a restricted maximum likelihood estimation. The field transplant LME models were analysed using the *lmer* function in the *lme4* R package. The laboratory LME and GLMM models were analysed using the *blmer* and *bgfmer* functions in the *blme* R package. We tested overall differences in mixed models using the ANOVA function in the *lmerTest* R package, except for the GLMM where we used the ANOVA function in the R package *car*. We tested post hoc differences in factorial predictors used in mixed models with a least-squares means approach using the *lsmeans* R package with Tukey's correction for multiplicity. In conjunction with the RMA regressions described earlier, we compared the slopes of the allometric mixed models with the R function *testIntInteractions* in the *phia* package using the *BH* correction for multiplicity. We calculated effect sizes (Standardised Mean Differences [SMD]) using the *SingleCaseES* R package, with the exception of survival in the laboratory experiment where we used log risk ratios calculated with the *fmsb* R package. We used effect sizes (and associated uncertainties) in addition to null hypothesis significance testing, because false-negative results can occur in ecotoxicological studies (Feckler et al., 2018). Reduced major axis model results are provided in Appendix S1: Tables C3 (Field Survey), C6 (Field experiment), and C9 (Lab experiment), with results from the latter two plotted in Figures C4 and C6, respectively. We also plotted initial and final body sizes to better understand growth relationships in the Field experiment (Appendix S1: Figure C5) and Lab experiment (Appendix S1: Figure C7). For full methods details, see Appendix S1. All statistical analyses were performed using R 3.6.2 (R Core Team, 2019).

3 | RESULTS

3.1 | Field survey

We used quantitative benthic samples to investigate demographic features of gammarid populations from locations above (US) and

below (DS) WWTPs at six study sites over 2 years (2013 and 2014). In the three sites surveyed in 2013, abundances of juvenile gammarids were greatly reduced at the DS location compared to the US location ($\chi^2_1 = 275, p < 0.001$; Figure 2). In particular, the number of small individuals (i.e. < 4 mm) decreased considerably at the DS locations (Figure 2, Appendix S1: Figure C1), whilst adults (≥ 7 mm) showed no changes (Figure 2, Appendix S1: Figure C2). However, gammarid density responses were inconsistent across all six study sites surveyed over the 2 years (Table 2). The Standardised Mean Difference (SMD) in juvenile abundances between the US and DS locations was negative (-0.53), but the difference was statistically

non-significant ($p = 0.37$). For adults, the SMD was positive (0.28) and non-significant ($p = 0.84$). Juvenile gammarids were more abundant than adults at both sampling locations, but the SMD between juveniles and adults was much stronger at the US location ($2.39, p < 0.001$) than the DS location ($0.30, p < 0.001$).

Across all six sites, proportions of adult *G. fossarum* and *G. pulex* differed between sampling locations whereby *G. pulex* was relatively more abundant at the DS location ($\chi^2_1 = 6.19, p < 0.05$). Increased proportions of adult *G. pulex* at the DS location were observed in the 2014 sites, despite *G. fossarum* being the dominant species ($\chi^2_1 = 21.2, p < 0.001$). At the 2013 sites, the difference in

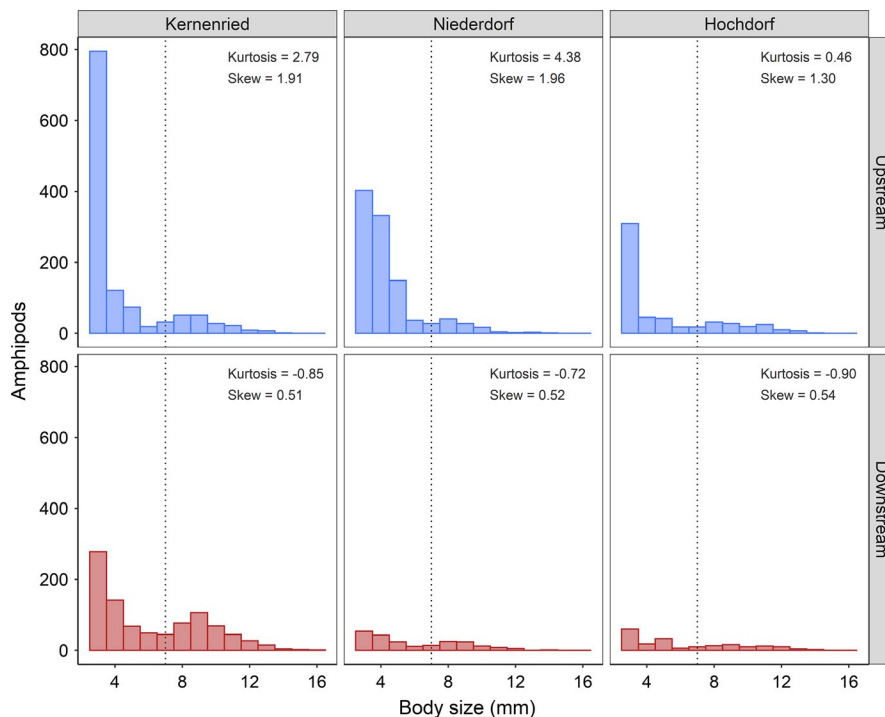


FIGURE 2 Size structure of amphipod populations (*Gammarus* spp.) in three Swiss streams where individual counts per size class (mm) are shown at locations upstream (blue) and downstream (red) of a wastewater outfall. The dotted lines indicate the division between juveniles (< 7 mm) and adult gammarids (≥ 7 mm). See Appendix S1 for greater detail in juvenile (Figure C1) and adult (Figure C2) populations [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Mean densities (individuals/m² \pm 1 SD) of juvenile and adult gammarid amphipods at sampling locations above (Upstream) and below (Downstream) wastewater outfalls in six Swiss streams sampled Autumn 2013 and 2014. The effect size (SMD \pm 1 SE) is shown for each life stage. Regions are defined according to the Swiss Modular Stepwise Procedure (Schaffner et al., 2013): J, Jura; PI, Swiss Plateau

Year	Site	Region	Juvenile (< 7 mm)		Adult (≥ 7 mm)	
			Upstream	Downstream	Upstream	Downstream
2013	Kernenried	PI	5,715 \pm 4,179	2,617 \pm 901	1826 \pm 1,401	3,166 \pm 928
	Niederdorf	J	4,637 \pm 1706	642 \pm 674	1876 \pm 1,126	735 \pm 454
	Hochdorf	PI	2,206 \pm 558	486 \pm 793	1,247 \pm 556	723 \pm 521
2014	Villeret	J	4,450 \pm 3,241	5,254 \pm 3,205	1,580 \pm 733	2,362 \pm 1598
	Knonau	PI	228 \pm 175	994 \pm 1,215	156 \pm 94	935 \pm 1,154
	Val-de-Ruz	J	957 \pm 900	1,072 \pm 904	542 \pm 295	371 \pm 376
All sites			2,647 \pm 2,886	2043 \pm 2,457	1,056 \pm 942	1,329 \pm 1,370
Excluding Val-de-Ruz ^a			3,130 \pm 3,081	2,320 \pm 2,694	1,203 \pm 1,013	1602 \pm 1,431
Effect size			-0.53 ± 0.51		0.28 ± 0.67	

^aThis site was subsequently found to have an upstream subsurface connection with a wastewater discharge (Burdon et al., 2019), so it was excluded from statistical tests.

species composition between US and DS locations meant that overall, adult gammarid size was significantly larger downstream of the WWTPs ($\chi^2_1 = 11.3, p < 0.01$), because adult *G. fossarum* were smaller than *G. pulex* ($\chi^2_1 = 155, p < 0.001$). There was no significant difference in mean sizes of adult *G. fossarum* between US and DS locations (0.26, $p = 0.50$). Likewise, mean sizes of adult *G. pulex* did not differ between locations (0.31, $p = 0.83$). Sex ratios did not differ, despite large numbers of intersex individuals at all sampling locations (Appendix S1: Table C1). The slope of the body size–fecundity relationship for gravid female gammarids became flatter at the DS location, and gravid females of *G. pulex* were generally larger at the US locations (Appendix S1: Figure C3). For more field survey results including effect sizes, see Appendix S1: Table C2.

3.2 | Field transplant experiment

We conducted a field transplant experiment at the study site in Knonau (Canton Zürich) where we took upstream gammarids

(juveniles, adult *G. fossarum* and *G. pulex*) and placed them in cages at locations above (US) and below (DS) the WWTP outfall. There was a significant log-linear increase in leaf consumption rates (% change in leaf area/day) with increasing gammarid body size, but not temperature ($F_{2,80} = 20.9, p < 0.001, R^2_{\text{adj}} = 32.7\%$, Table 3). Increasing gammarid body size led to greater temperature-corrected consumption at the DS location ($p < 0.05$; Table 4; Figure 3), whereas this positive relationship was non-significant at the US location ($p = 0.13$). Adult *G. pulex* consumed more than *G. fossarum* at the DS location (1.07, $p < 0.01$); a difference probably explained by the larger size of *G. pulex* (Appendix S1: Table C5). In contrast, consumption by adult *G. pulex* compared to *G. fossarum* did not differ at the US location (1.10, $p = 0.30$). The difference in consumption between US and DS locations was statistically non-significant for juvenile gammarids (−0.65, $p = 0.47$), adult *G. fossarum* (−0.11, $p = 0.58$), and adult *G. pulex* (0.29, $p = 0.16$).

There was a significant log-linear decrease in relative growth rates (Eq.2) with body size but not temperature ($F_{2,80} = 9.45, p < 0.001, r^2_{\text{adj}} = 17.1\%$, Table 3). After temperature corrections, growth

TABLE 3 Parameter estimates with associated confidence intervals (95%), *t*-values, and *p*-values for the log-linear models describing the main effects of body size (mm) and temperature on consumption and growth rates of gammarid amphipods in the field transplant experiment. Parameters correspond to those listed in Eq.4, where R_0 is the process rate at temperature T_0 , b_R is the allometric exponent, and $-E_R$ is the activation energy. The negative slope for E_R comes from the inverse temperature predictor $1/kT_R - 1/kT_0$ used in the linearised Arrhenius function derived from Eq.4. The negative b_R value for growth matches the direction of the $-1/4$ power scaling coefficient described by West et al. (2001) for size-specific activity (relative growth Eq.2 accounts for size by using the difference in log-transformed sizes)

Response variable	Parameter	Estimate	95% CI	<i>t</i> -value	<i>p</i> -value
Consumption	R_0	−0.203	−0.457–0.051	−1.591	0.116
(ln[x + 1] % Δ area/day)	b_R	0.392	0.271–0.513	6.439	<0.001
	$-E_R$	−0.301	−1.872–1.269	−0.382	0.704
Relative growth	R_0	0.024	0.015–0.033	5.455	<0.001
(Δ ln [mm]/day)	b_R	−0.009	−0.013– −0.005	−4.295	<0.001
	$-E_R$	−0.022	−0.076–0.032	−0.812	0.419

TABLE 4 Results from linear mixed-effects (LME) models testing how individual gammarid amphipod performance indicators scale with standardised log-transformed body size (mm) upstream and downstream of a wastewater outfall (field transplant experiment). Performance indicators: consumption (% change in leaf area/day) and relative growth (change in ln length [mm]/day). Both indicators were divided by the universal temperature dependence $e^{E_R T / k T_R T_0}$ (Gillooly et al., 2001) to account for warming associated with the WW outfall (see Section 2.6). CI, confidence interval. Juvenile gammarids are included in the models as the reference level. r_m^2 , marginal r^2 ; r_c^2 , conditional r^2

Performance indicator	Predictors	Estimate	95% CI	<i>p</i> -value	r_m^2	r_c^2
Consumption	(Intercept)	0.617	0.336–0.898	<0.001	37.6%	37.9%
(ln[x + 1] % Δ area/day)	Adult <i>G. fossarum</i>	−0.098	−0.454–0.258	0.590		
	Adult <i>G. pulex</i>	0.034	−0.407–0.474	0.881		
	Size × Upstream	0.150	−0.046–0.346	0.134		
	Size × Downstream	0.217	0.026–0.409	0.026		
Relative growth ^a	(Intercept)	−0.067	−0.157–0.022	0.140	33.9%	41.0%
(Δ ln [mm]/day)	Adult <i>G. fossarum</i>	0.163	0.052–0.275	0.004		
	Adult <i>G. pulex</i>	0.200	0.062–0.337	0.004		
	Size × Upstream	−0.139	−0.199– −0.079	<0.001		
	Size × Downstream	−0.116	−0.175– −0.057	<0.001		

^aParameter estimates and 95% CI for growth ($\times 10^4$)

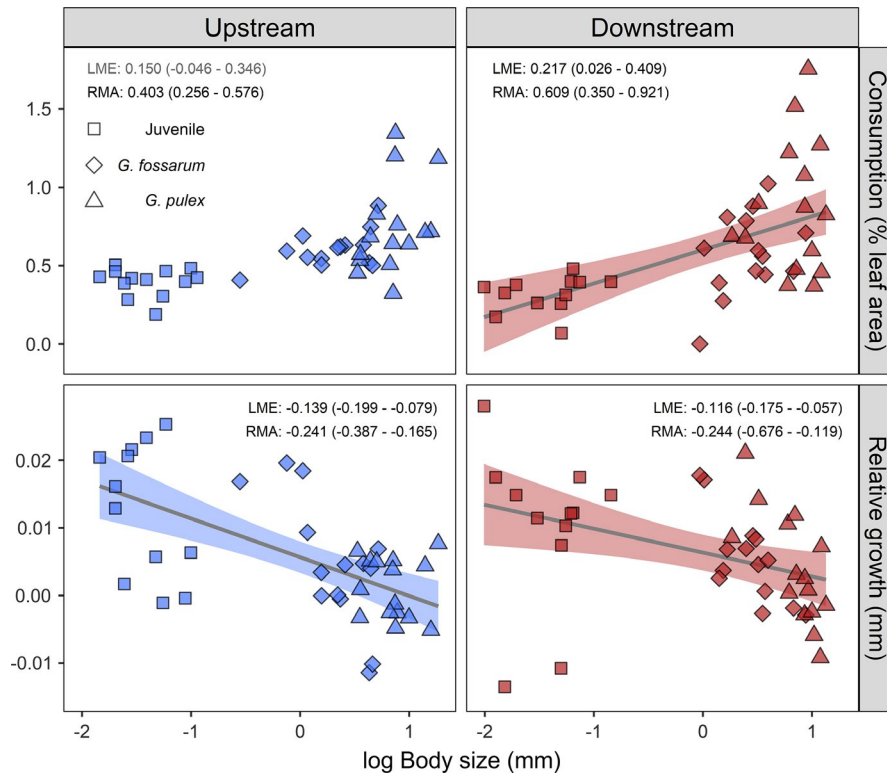


FIGURE 3 Results from the field transplant experiment showing how individual amphipod (*Gammarus* spp.) indicators of performance (consumption and growth) scale with body size (mm) upstream (blue) and downstream (red) of a wastewater outfall. Performance indicators: consumption ($\ln[x + 1]$ % change in leaf area/day) and relative growth rates (change in \ln length [mm]/day). Both indicators were divided by the universal temperature dependence $e^{E_R T / k T_R T_0}$ (Gillooly et al., 2001) to account for warming associated with the WW outfall (see Section 2.6). Body size (mm) is natural log-transformed and standardised. Slope parameter estimates (95% confidence interval in parentheses) for consumption and growth ($\times 10^1$) from the linear mixed-effects models (LME; Table 3) and the reduced major axis (RMA) regressions are provided for comparisons (in black, significant at $\alpha = 0.05$; grey, non-significant) [Colour figure can be viewed at wileyonlinelibrary.com]

rates were significantly lower in adults compared with juveniles, reflecting a significant decrease with increasing gammarid body size at both locations (Figure 3, Table 4). The body size-growth relationship did not differ between the US and DS locations ($\chi^2_1 = 2.06$, $p = 0.15$). The difference in growth between US and DS locations was statistically non-significant for juvenile gammarids (-0.20 , $p = 0.49$), adult *G. fossarum* (-0.10 , $p = 0.48$), and adult *G. pulex* (0.33 , $p = 0.39$).

Mortality was negligible, with only five individuals dying (6% of all gammarids used) and no obvious trends (Appendix S1: Table C4). More field experiment results including effect sizes and test statistics are reported in Appendix S1: Tables C5 and C7, respectively.

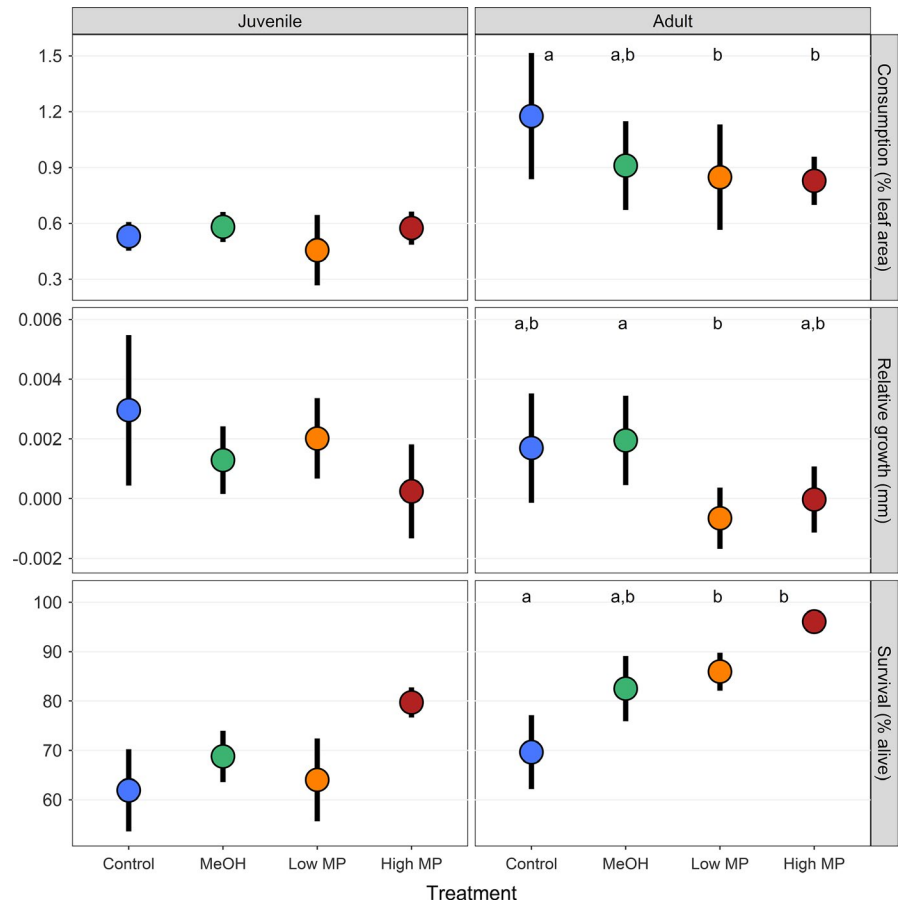
3.3 | Laboratory experiment

We tested the effects of a realistic MP mixture at two concentrations (Table 1) on juvenile and adult *G. fossarum* in a laboratory experiment. Leaf consumption rates (% change in leaf area/day) varied with life stage and body size (Figure 4). Adult gammarids showed decreased consumption in MP treatments compared to the Control (Figure 4), with the negative SMD largest in the Low MP treatment (-0.34 , $p < 0.01$), followed by the High MP treatment (-0.27 , $p < 0.05$). However, both differences became non-significant when including body size as a

covariate (Low MP, $p = 0.08$; High MP, $p = 0.22$). Overall, juvenile gammarids consumed less than adults ($\chi^2_1 = 34.6$, $p < 0.001$). However, there were no statistically significant MP effects on juvenile consumption (Figure 4). Explicitly testing the relationship between body size and consumption across treatments demonstrated how this trait positively influenced resource acquisition (Figure 5, Table 5). However, the slopes of the body size-consumption relationship did not statistically differ across treatments after corrections for multiplicity (Appendix S1: Table C10).

Adult growth rates were significantly lower than juvenile growth rates when gammarids were exposed to the Low MP treatment (-0.91 , $p < 0.05$); a difference which became highly non-significant when body size was included as a covariate ($p = 0.89$). In general, the negative effects of MPs on gammarid growth rates were equivocal (Figure 4). Overall, gammarids grew more in the Control than the High MP treatment (-0.42 , $p < 0.05$). Adults in the MeOH treatment grew more than those in the Low MP treatment (-0.65 , $p < 0.05$). However, adult growth in the Control did not differ significantly from either the Low MP (-0.57 , $p = 0.12$) or High MP treatments (-0.42 , $p = 0.31$). Likewise, juvenile growth in the Control did not differ significantly from either the MeOH (-0.38 , $p = 0.55$), Low MP (-0.21 , $p = 0.89$), or High MP treatments (-0.61 , $p = 0.17$).

FIGURE 4 Results from the laboratory experiment showing mean individual performance (\pm 95% CI) of juvenile and adult gammarid amphipods (*Gammarus fossarum*). Consumption (% change in leaf area/day); Growth (change in ln length [mm]/day); survival (mean % alive over 21 days). Treatments: Control, MeOH (an addition of methanol as a technical control), Low MP and High MP micropollutant (MP) concentrations. Letters (a, b) indicate post hoc differences in treatment effects including body size as a covariate (except consumption where no statistically significant post hoc differences were detected with size included in the mixed model) [Colour figure can be viewed at wileyonlinelibrary.com]



Growth decreased with increasing gammarid body size in the Control and Low MP treatments (Figure 5, Table 5). However, the slopes of the body size-growth relationship did not differ significantly in these two treatments ($\chi^2_1 = 1.06, p = 0.45$). The slope estimate from the RMA regression of the MeOH treatment was more similar to the Control (Figure 5). However, the LME models indicated that growth did not correlate with body size in the MeOH and High MP treatments (Figure 5, Table 5).

Overall, the survival of adult gammarids (68%) was greater than that of juveniles (50%) at the end of the experiment ($\chi^2_1 = 8.43, p < 0.01$; Figure 4). Juvenile survival was reduced in the Low MP treatment compared to adult survival with a negative log risk ratio of -0.61 ($p < 0.01$). This result was also reflected in the statistically significant size-survival relationship in the Low MP treatment (Figure 5, Table 5). The difference between adult and juvenile survival in the Low MP treatment remained after accounting for body size ($p < 0.05$). Juvenile survival did not differ statistically from adult survival in the High MP treatment ($-0.22, p = 0.09$).

Surprisingly, overall gammarid survival was greatest in the High MP treatment (81%), and significantly lower in the Control (42%, $p < 0.001$) and MeOH treatments (60%, $p < 0.05$). Adult gammarid survival was greater with positive log risk ratios in the Low MP (0.51, $p < 0.05$) and High MP treatments (0.66, $p < 0.01$) relative to the Control. In contrast, juvenile survival in the High MP treatment did

not differ statistically from the Control (0.60, $p = 0.09$). There was no difference in juvenile survival between the Control and Low MP treatments (0.06, $p < 0.999$). Additional laboratory experiment results are reported in Appendix S1, including effect sizes (Tables C8, C12), test statistics (Table C11), and survival curves (Figure C8).

4 | DISCUSSION

Understanding how environmental stressors affect individual fitness and population structure is important for explaining patterns in biodiversity and ecosystem functioning (Galic et al., 2018). We tested the influence of treated municipal WW and MPs on gammarid amphipods using multiple approaches and found that the effects were context and life stage dependent. Firstly, our field surveys showed that densities of juvenile gammarids were reduced below WW inputs in three of the six study sites (similar to Ladewig et al., 2005). The field transplant experiment showed that at the individual level, the relationship (slope) between leaf consumption and gammarid body size was steeper in the presence of WW, with decreased consumption by juveniles and increased consumption by adults contributing to this change. In the laboratory experiment, exposure of MPs to individual *G. fossarum* on average increased survival, but reduced growth and consumption relative to controls. We also saw

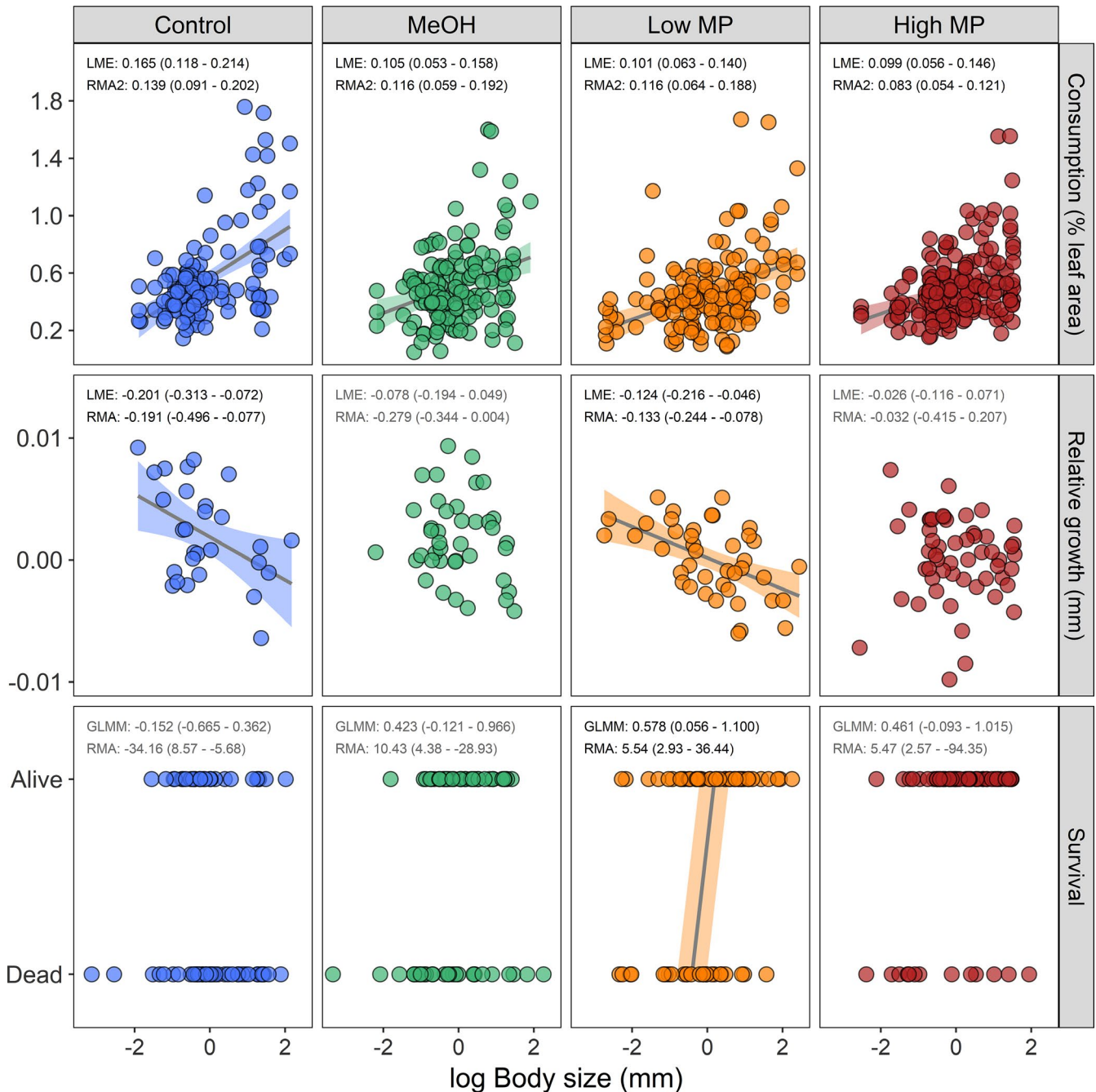


FIGURE 5 Results from the laboratory experiment showing the relationship between individual amphipod (*Gammarus fossarum*) body size and performance indicators: consumption ($\ln[x + 1]$ % change in leaf area/day), growth (change in \ln length [mm]/day), and survival (after 21 days). Body size (mm) is natural log-transformed and standardised. Treatments: Control, MeOH (an addition of methanol as a technical control), Low MP and High MP micropollutant (MP) concentrations. The slope parameter estimates (95% confidence intervals in parentheses) for the mixed models (linear mixed-effects [LME] and generalised linear mixed-effect [GLMM]; Table 5) and the reduced major axis (RMA) regressions are provided for comparisons (in black, significant at $\alpha = 0.05$; grey, non-significant). Relative growth: LME estimates $\times 102$; RMA estimates $\times 101$ [Colour figure can be viewed at wileyonlinelibrary.com]

evidence of asymmetries in stress responses across life stages, where differences in the body size–response relationship of consumption were influenced by reductions in adult feeding when exposed to chemical pollution. In contrast, gammarid juveniles had significantly greater mortality than adults in response to MPs, evidenced by a positive slope in the relationship between body size and survival.

4.1 | Gammarid responses to chemical pollution in the environment

Our main prediction was that asymmetric sensitivity between gammarid life stages (i.e. juveniles vs. adults) to chemical pollution should affect population size, demography, and individual performance. We hypothesised that juveniles are more sensitive to chemical pollution

TABLE 5 Results from linear mixed models testing how individual gammarid amphipod performance indicators scale with standardised log-transformed body size (mm) in different water treatments (laboratory experiment). Performance indicators: % consumption ($\ln[x + 1]$ % change in leaf area/day), growth (change in \ln length [mm]/day), and survival (after 21 days). MeOH, methanol (Technical control); MP, micropollutants. Adult gammarids are in the consumption model as the reference level. r_m^2 , marginal r^2 ; r_c^2 , conditional r^2

Performance indicator	Predictors	Estimates/Odds ratios	95% CI	p-value	r_m^2	r_c^2
Consumption	(Intercept)	0.496	0.378–0.610	<0.001	12.1%	52.7%
($\ln[x + 1]$ % Δ area/day)	Juvenile	0.012	–0.042–0.079	0.725		
	Size \times Control	0.165	0.118–0.214	<0.001		
	Size \times MeOH	0.105	0.053–0.158	<0.001		
	Size \times Low MP	0.101	0.063–0.140	<0.001		
	Size \times High MP	0.099	0.056–0.146	<0.001		
Relative growth ^a	(Intercept)	0.080	0.022–0.141	0.387	9.2%	31.2%
($\Delta \ln$ [mm]/day)	Size \times Control	–0.201	–0.313––0.072	0.001		
	Size \times MeOH	–0.078	–0.194–0.049	0.212		
	Size \times Low MP	–0.124	–0.216––0.046	0.005		
	Size \times High MP	–0.026	–0.116–0.071	0.588		
Survival	(Intercept)	0.500	–0.026–1.027	0.063	12.8%	32.9%
(alive or dead)	Size \times Control	–0.152	–0.665–0.362	0.562		
	Size \times MeOH	0.423	–0.121–0.966	0.127		
	Size \times Low MP	0.578	0.056–1.100	0.030		
	Size \times High MP	0.461	–0.093–1.015	0.103		

^aParameter estimates and 95% CI: relative growth $\times 10^2$

(Figure 1), and found some supporting evidence in our field survey and experiments. We posited that body size is an important determinant of individual performance, and a fundamental trait mediating sensitivity to pollution in gammarids. Other studies have demonstrated sensitivity of juvenile gammarids to WW-borne MPs, such as the pyrethroid insecticide deltamethrin (Adam et al., 2010) and heavy metal cadmium (Alonso et al., 2010). This susceptibility may be due to smaller-bodied organisms being more sensitive to toxicity (Buchwalter & Luoma, 2005). Their higher specific metabolism, coupled with a greater surface to volume ratio, may increase uptake rates of chemicals and subsequent toxic effects, as demonstrated empirically (Wang & Zauke, 2004) and mechanistically described by dynamic energy budget theory (Kooijman, 2010). Higher growth and resting metabolic rates impose a cost which may limit the *scope for activity* in juveniles; here increasing metabolism to help process and eliminate toxic MPs (Calow & Sibly, 1990).

Life-stage dependent effects arising through developmental ontogeny may account for increased mortality not explained by differences in body size alone. For example, the integument of juveniles can be more permeable than in adults as they develop (Verriopoulos & Moraitouapostolopoulou, 1982). In gammarids and other crustaceans, calcium is used by individuals in the post-exuvial period to mineralise the new cuticle (Graf & Meyran, 1983), meaning the calcium accumulation pathway can lead to *accidental active uptake* of contaminants (Wright, 1980). More frequent post-exuvial mineralisation of new cuticle, owing to the higher growth and moulting rate of juvenile gammarids, could therefore exacerbate uptake of

toxicants (Pöckl, 1992). Thus, growth rates and moulting may be an important life history feature further mediating sensitivity to toxicants (McCahon & Pascoe, 1988; see also Section 4.2).

Alternatively, as size is strongly correlated with age, it may be age per se that is the ultimate driver of different responses in juveniles and adults. Size and age are correlated, although phenotypic plasticity (e.g. inhibited growth and development) can complicate the relationship (Pöckl et al., 2003). One potential mechanism related to age is desensitisation, where individual stress tolerance increases with exposure duration, thus with age (Rozin & Schiller, 1980). However, there is limited experimental evidence for this mechanism (Rozin et al., 1979), and it would only be relevant in the field studies, since the gammarids in the laboratory experiment were presumed to come from an unimpacted environment. Another aspect of age and ontogeny relates to the microbiome of organisms which helps sustain host fitness and may confer pollution tolerance (Fraune & Bosch, 2010, Claus et al., 2016, Koskella et al., 2017). For instance, as juvenile crustaceans (e.g. isopods) develop, they acquire endosymbionts from the environment (Horváthová et al., 2015), meaning that chemicals that disrupt the microbiome may exert negative indirect effects on hosts. However, the potential role of the microbiome in gammarid fitness and chemical tolerance remains uncertain with no effects reported for *G. pulex* in an endosymbiont *knock-out* study (Zimmer & Bartholmé, 2003).

In general, environmental stress can increase maintenance costs and reduce resource assimilation, meaning fewer resources are available for growth and reproduction (Maltby, 1999). Thus, negative

effects of chemical pollution on adults (e.g. via reduced female fecundity) may contribute to reduced juvenile densities at WW-impacted locations. Our field transplant experiment indicated that the larger adult *G. pulex* consumed more than the smaller *G. fossarum* in the presence of WW only (i.e. at the downstream site), but decreased consumption by juveniles also contributed to a steeper allometric relationship. Assuming asymmetric responses, increased consumption by adults could be indicative of higher metabolic rates and stress (Barber et al., 1990). Another potential stress pathway affecting adult gammarids could be mediated through mating behaviour, where pollution can disrupt mate guarding behaviour (Pascoe et al., 1994) or change sex ratios through the effects of synthetic hormones (Gross et al., 2001). We did not assess pre-copulatory behaviour, and there was little evidence for changes in sex ratios, despite large numbers of intersex individuals at all sampling locations. Chemical pollution, nutrients, and/or elevated temperatures could also cause changes in the timing of key life-history events, as shown for *G. pulex* with a breeding peak shift into winter at WW-impacted sites (Hobrough, 1973; see also Ladewig et al., 2005).

Female body size is a strong determinant of fecundity (brood size) which may affect juvenile gammarid densities in the wild (Sutcliffe, 1993). Peschke et al. (2014) reported that gammarids from a WW-impacted stream in Germany had female-biased sex ratios but reduced female fecundity. We saw some evidence for reduced *Gammarus* spp. fecundity at the downstream sites with the body size–fecundity relationship becoming flatter (i.e. larger individuals less fecund below the WWTP), and gravid females of *G. pulex* were generally smaller at downstream locations, indicating potential impacts on individual fitness and population growth (Peters, 1983). Alternatively, egg development and hatching success can affect juvenile gammarid densities directly (Pöckl & Humpesch, 1990). For example, MPs may affect secondary vitellogenesis or embryonic development (Geffard et al., 2010). Thus, if chemical stress induces energy limitations on females (e.g. through increased metabolic demands and decreased resource quality; Sokolova, 2013), egg quality (e.g. egg-yolk precursor materials such as vitellogenin proteins) may be affected, warranting proteogenomic analyses (Trapp et al., 2014). Ganser et al. (2019) reported no change in vitellogenin levels of *G. fossarum* when exposed to WW, but only males were tested. The potential for reduced fecundity in response to chemical pollution indicated by our study may have population-level consequences (Hoffmann & Hercus, 2000), yet this endpoint is rarely used in ecotoxicological studies.

Another possible pathway of chemical stress effects on individuals and populations could arise via biotic interactions, which can modulate the ecological impacts of chemical stressors (Halstead et al., 2014). For example, WW could alter competition via intra or interspecific interactions between juvenile and adult gammarids. *G. pulex* is considered a habitat generalist with broader niche requirements than *G. fossarum*, making it generally more tolerant to environmental stress (Alonso et al., 2010; Peeters et al., 1998). In our field survey, *G. pulex* became relatively more abundant at WW-impacted locations, potentially indicating stronger competition with

G. fossarum. Similarly, *G. fossarum* dominated at the 2014 sites, potentially explaining the discrepancy in juvenile responses. Pollution-mediated changes to interspecific interactions might reflect *apparent competition* where increases in shared resources can adversely impact a common predator, parasite, or pathogen (Holt, 1977). In the context of our study, higher abundance of adult gammarids at WW-impacted sites (Ladewig et al., 2005) could result in adverse impacts on juveniles as gammarids frequently cannibalise smaller congeners (MacNeil et al., 1997, 1999). Alternatively, changes in predation by higher consumers such as *Cottus* spp. (Cottidae) can drive size structuring in gammarid populations (Glazier et al., 2011). Indirect biotic interactions are the most tenuous of our proposed mechanisms, but warrant targeted experiments in future research.

4.2 | Experimental evidence for complex responses to MPs

Wastewater effluent is a complex mix of MPs, nutrients, and microbes (Burdon, Bai, et al., 2020). To disentangle differences in sensitivity to MPs between life stages, we exposed adult and juvenile *G. fossarum* to two different concentrations of a realistic MP mixture. This mixture included the pesticide diazinon, a known toxicant to gammarids with lethal concentrations for *Gammarus* spp. (including *G. fossarum*) ranging from 0.2–30 µg/L (Mayer & Ellersieck, 1986; Nyman et al., 2014). Thus, the diazinon concentrations used in our experiment (0.06–0.45 µg/L) could alone cause chronic to acute effects in gammarids. However, contrary to our expectations, adult gammarids experienced the lowest mortality in the High MP (13%) and Low MP (25%) treatments relative to the technical control (MeOH; 35%) and Control treatments (55%), with similar results observed with juvenile gammarids. Five mechanisms might explain greater survival in the MP treatments, with the first four involving biological responses to the MP mixtures in general. The fifth mechanism involves antagonistic interactions among specific compound classes with different modes of action used in the mixtures and is covered in a separate section (see Section 4.3).

First, the *growth-mediated stress* hypothesis posits that increased growth rates add stress during moulting, contributing to greater mortality. Hence, control treatments could have caused increased mortality because gammarids were growing faster and more frequently entering the sensitive post-moult phase (McCahon & Pascoe, 1988). This hypothesis is important for size dependency because, according to allometric theory (e.g. MTE, dynamic energy budget) intra-specific growth rates should scale with body size (i.e. mass) raised to the power of 0.6–1 (Brown et al., 2004; van der Meer, 2006; West et al., 2001). Thus, faster specific growth rates could place juveniles at greater risk from chemical stressors (see also Section 4.1), because increased growth, coupled with higher specific metabolic rates impose a cost on the ability of an individual to metabolise toxicants (Calow & Sibly, 1990).

Secondly, according to the *hormesis* hypothesis, low-dose stimulation (i.e. pollutants at low concentrations) can cause

overcompensation responses (e.g. increased survival), leading to improved performance relative to *no stimulation* or *high stimulation* scenarios (Calabrese, 2001). In our experiment, both MP treatments could be regarded as low-dose stimulation for adults (survival in High and Low MP treatments was higher than in the Control), whereas for juveniles only individuals in the High MP treatment showed this pattern. However, hormesis influences other responses that contribute to survival, and normally is difficult to detect because of small effect sizes (Calabrese, 2008).

Thirdly, the *pathogen-release* hypothesis posits that naturally present pathogens could be negatively affected by MPs, thus reducing a source of natural mortality for the hosts (cf. Zimmer & Bartholmé, 2003). The biocide triclosan, a commonly found MP in WW effluent and a component of our MP mixture, can exert negative effects on protozoans, which include common microsporidian parasites of gammarids (Gao et al., 2015; Rowley & Powell, 2007). Another indirect pathway could be mediated through reduced detrital resource quality, which perhaps paradoxically can decrease parasitism rates in *G. pulex* (Sanchez-Thirion et al., 2019). Thus, constituents of our MP mixture may have had negative direct and indirect effects on natural enemies, potentially contributing to gammarid survival in the MP treatments.

Alternatively, methanol used as a solvent (in the MP mixtures and the technical control) may have provided a labile carbon source for microbes, thus enhancing food resources for gammarids. Methylophilic bacteria (Dixon et al., 2013; Halsey et al., 2012) could cause methanol to have strong *subsidy* effects on freshwater organisms even at low concentrations (Taylor et al., 2016). We found that the Low MP treatment potentially had stronger negative effects on consumption and growth than the High MP treatment, indicating there may have been a concentration-dependent compensatory effect from the methanol helping to *mask* or *dampen* MP effects (i.e. an antagonistic interaction). However, the *subsidy* mechanism is seemingly contradicted by lower adult gammarid growth rates in the MP treatments, although reduced growth may reflect greater individual maintenance costs due to toxic stress. This effect would be consistent with the *principle of allocation*, where there is a trade-off between scope for growth and metabolism (Calow & Sibly, 1990).

4.3 | Interactions in complex MP mixtures

The interaction of environmental conditions and toxic pollutants has been widely studied in different organisms (Holmstrup et al., 2010; Macaulay et al., 2021; Salo et al., 2018). For instance, the toxicity of chemicals acting on metabolic processes generally increase at higher temperatures due to greater organismal metabolic rates and stimulated toxicokinetic parameters (Heugens et al., 2001). In contrast, the ecotoxicology of complex MP mixtures at environmentally relevant concentrations remains poorly understood (Backhaus, 2014), particularly at lower concentration ranges (Orton et al., 2014). Synergistic effects (greater than the sum of individual effects) may

be a feature of mixtures containing groups of pesticides known to interfere with metabolic degradation of other MPs, such as the cholinesterase inhibitors or azole fungicides (Cedergreen, 2014). Most insecticides target the nervous system mediated by acetylcholine neurotransmitters, such as the carbamate and organophosphates groups which disrupt the enzyme acetylcholinesterase whilst neonicotinoids act as antagonists to acetylcholine receptors (Nyman et al., 2013; Xuereb et al., 2009). Consequently, carbamate pesticides can have synergistic effects on insects when acting in combination with insect repellents (i.e. DEET) containing N,N-diethyl-m-toluamide (Pennetier et al., 2005). Furthermore, complex toxic interactions on oxidative stress, detoxification and neurotoxicity biomarkers have been recorded in amphipods and insect larvae exposed to combined pesticides (Corbel et al., 2006; Demirci et al., 2018; Rösch et al., 2017). However, we only included one insecticide (the organophosphate diazinon) and one azole fungicide (the triazole tebuconazole) in our MP mixture, and in general, synergistic interactions may be relatively uncommon in mixtures of pesticides and other MPs including metals and antifoulants (Cedergreen, 2014).

Other important consequences of MP exposure may include behavioral effects associated with psychoactive drugs. Antidepressants of the selective serotonin reuptake inhibitor (SSRI) class such as fluoxetine (commonly known by the brand name Prozac) have been shown to reduce activity rates in *G. pulex* (De Lange et al., 2006), and gene expression of putative serotonergic pathways in amphipods indicates the potential for anxiety reduction (Bossus et al., 2014). One of the few studies to have investigated interactive effects of antidepressants and pesticides (the neonicotinoid insecticide imidacloprid) showed antagonistic effects (less than the sum of individual effects) on activity-rate responses in the crustacean *Daphnia magna*, which the authors hypothesised might derive from fluoxetine's inhibition of imidacloprid's target receptor (Schlüssel & Leininger, 2019). By potentially inhibiting pesticide modes of action, in addition to reducing activity and anxiety, exposure to antidepressant drugs could be coupled with a reduction in metabolic demands. These possible effects provide an alternative hypothesis as to why gammarids showed greater survival when exposed to MP mixtures including the SSRI citalopram in our study. To summarise, although several studies have suggested that toxic effects of mixtures act additively (Altenburger et al., 2018; Oliveira et al., 2017; Watanabe et al., 2016), mixture toxicity models based on concentration addition and independent action fail to consider interactions occurring at the toxicokinetic or toxicodynamic level (i.e. the level of uptake and biotransformation at the receptor site; Backhaus, 2014). Further research is required to fully understand the ecotoxicology of mixtures including pharmaceuticals in environmentally realistic settings.

5 | CONCLUSIONS

Improving our understanding of how organismal body size responds to global change drivers such as chemical pollution is essential for predicting impacts on food webs and ecosystem

functioning. We found that the effects of chemical pollution on gammarid amphipods may be life stage and size dependent. However, the idiosyncratic results from our field and laboratory studies further highlight the potential for biological and environmental contingencies in organismal responses to human pressures (Burdon, Ramberg, et al., 2020; Burdon et al., 2016). Predicting higher-level impacts of MPs necessitates an understanding of multiple stressor responses (Jackson et al., 2016), as chemical pollutants frequently interact with additional threats, such as warming (Salo et al., 2018). A recent simulation of multiple stressor effects across three levels of biological organisation (i.e. individual performance, population attributes, and ecosystem processes) in a stream amphipod model found that interactive effects of multiple stressors at the individual level were mostly antagonistic, whereas population and ecosystem-level responses to stressor combinations were synergistic (Galic et al., 2018). Whilst stressor combinations rarely act additively (Jackson et al., 2016), negative stressor effects may be magnified at higher levels of biological organisation (Galic et al., 2018), potentially contributing to *cryptic function loss* (i.e. where animal populations persist, but their functioning in impacted ecosystems is impaired; McConkey & O'Farrill, 2015). Thus, individual responses in our laboratory experiment may have underestimated the consequences of MPs for population abundances and ecosystem functions, as potentially indicated by reduced juvenile densities at some of our WW-impacted field sites. Nonetheless, cross-scale studies investigating environmental stress should account for individual trait variation, including body size, and population demographics encompassing different life stages (see also Debecker et al., 2016). As we have demonstrated here, allometric theory can help us examine stressor effects on underlying organismal biology, population demographics, and conceptually link ecotoxicology with macroecology.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

F.J.B., A.T., and K.R. designed the study; F.J.B. and A.T. collected field samples; A.T. conducted the experiments and invertebrate analyses;

F.J.B. analysed the data; F.J.B. led the manuscript writing with contributions from A.T. and K.R.

DATA AVAILABILITY STATEMENT

Data will be made available via the Dryad Digital Repository (Burdon, Taddei, & Räsänen, 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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