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Tandem action of natural and chemical stressors in stream ecosystems: insights from a population genetic perspective

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Abstract

Agricultural and urban land use has dramatically increased over the last century and one consequence is the release of anthropogenic chemicals into aquatic ecosystems. One of the rarely studied consequences is the effect of land use change on internal concentrations of organic micropollutants (OMPs) in aquatic invertebrates and its effects on their genotype diversity. Here, we applied population genetic and internal concentrations of OMPs analyses to determine evolutionary implications of chemical pollution on *Gammarus pulex* populations from a natural and two agricultural streams. Along 14 consecutive months sampled, 26 different OMPs were quantified in *G. pulex* extracts with the highest number, concentration, and toxic pressure in the anthropogenically stressed stream ecosystems. Our results indicate distinct internal OMP profiles and changes in both genetic variation and genetic structure in streams affected by anthropogenic activity. Genetic variation was attributed to chemical pollution whereas changes in the genetic structure were attributed to environmental disturbances, such as changes in discharge in the impacted stream ecosystems, which worked both independently and in tandem. Finally, we conclude that human-impacted streams are subjected to severe alterations in their population genetic patterns compared to non-impacted stream ecosystems.

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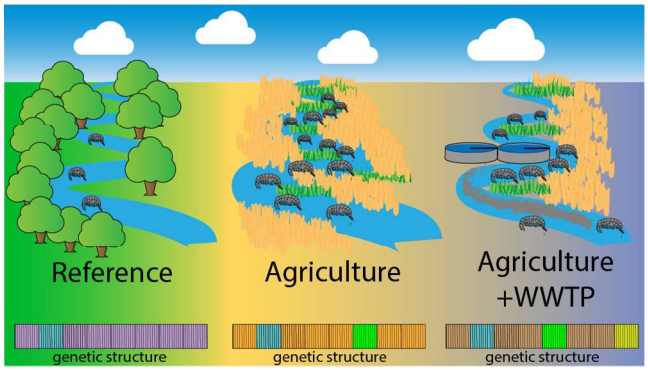
30 

Introduction

During the last century, the intensity of land use has dramatically increased due to human population growth.[1] As a consequence, agricultural and urban land uses have reduced or fragmented natural areas for wild species.[1] Agricultural land use is associated with application of fertilisers and pesticides, whereas urban land use acts as a source of potentially toxic compounds, such as pharmaceuticals and industrial chemicals.

To date, a strong relationship exists between land use and/or chemical pollution, and quantitative measures of biological responses in stream ecosystems. Once pollutants enter water bodies they may cause disruption of the endocrine system, protein malfunctioning, fluctuations in abundance and changes in both species diversity and distribution of aquatic organisms.[2,3] The magnitude of these effects will depend on the reactivity of the pollutant, the sensitivity of an organism, and duration and magnitude of the exposure. Recently, a shaping role was allocated to chemical pollution and particularly to organic micropollutants (OMPs) modifying the genetic structure of freshwater macroinvertebrates living along a land use gradient.[4]

Effects at genetic level may be more disruptive than individual−level effects. If clean−up processes are improved or the source of pollution is removed, metabolic and physiological responses of the organisms may return to normal or pre−polluted state, but genetic metrics may take many generations to return to a pre−polluted state.[5] Moreover, chemical pollution can affect both neutral and selective genetic regions favouring tolerant genotypes, bottlenecks and/or eliminating non−tolerant genotypes, altering the exchange of migrant individuals among populations and reducing effective population sizes.[5,6] The resulting populations after these processes could be populations with reduced census sizes and effective population sizes, where

genetic drift can act more rapidly, reducing the genetic variability (depleting rare alleles) as well as increasing population differentiation. Hence, assessment of individual–level effects alone may underestimate the real effect of chemical pollution on aquatic organisms.

There is an increasing awareness that the analysis of the internal concentration of pollutants in biota is key to a better understanding of the mechanisms behind the causal links between exposure and environmental effects.[7] Freshwater macroinvertebrates are known to accumulate OMPs,[8,9] equilibrating their internal concentrations to water concentrations and can thus exhibit distinct internal pollution profiles according to their spatial distribution.[10] For example, macroinvertebrates inhabiting streams that drain agricultural catchments may principally accumulate pesticides, while in urban streams, macroinvertebrates are more likely to accumulate pharmaceuticals and industrial chemicals. Internal pollution profiles may play different roles favouring genotypes and ultimately structuring the population genetics of freshwater macroinvertebrates from a land use perspective. It is well known that land use and chemical pollution can independently act as main drivers of biological patterns.[11,12] Nevertheless, there is scarce empirical evidence to support in tandem effects in macroinvertebrate freshwater populations long−term exposed to chemical pollution. This is especially true for chemicals at trace concentrations such as OMPs in stream ecosystems affected by agricultural and wastewater treatment plant (WWTP) activities.

The overarching goal of our study was to gain insights into the long−term effects of land use and the effect of resultant chemical pollution by OMPs on neutral patterns of genetic variation in the common freshwater macroinvertebrate *Gammarus pulex*. *G. pulex* has relevant ecological roles breaking down coarse particulate organic matter[13] as well as in food webs linking lower trophic levels to fish.[14] In many freshwater communities, *G. pulex* can represent the dominant

76 macroinvertebrate in terms of biomass and relative abundance.[14,15] This species spends much of
77 its life in contact with sediments, providing a continuous exposure to both hydrophilic water–
78 based and hydrophobic sediment−based pollutants.[10] This is therefore, considered an optimal
79 model organism for evolutionary toxicology studies and body burden monitoring. Indeed, this
80 has already been used as a model organism for assessing both adverse effects,[16,17] and uptake of
81 OMPs under laboratory[18] and field conditions.[8,9] Additionally, due to its important ecological
82 position, any effect in field populations may also lead to broader negative consequences on
83 ecosystem functioning.

84 To this end, three major hypotheses were tested: (i) stream ecosystems under agricultural and
85 urban land uses have distinct internal OMP profiles, with higher concentrations and frequency of
86 OMPs in *G. pulex* inhabiting anthropogenically impacted streams. (ii) *G. pulex* populations
87 under long-term exposure to OMPs have lower genetic variability due to genetic erosion, and
88 (iii) long−term exposure to OMP's result in more complex genetic structures than populations
89 living in non−impacted stream ecosystems.

90 Materials and Methods

91 Study area and sampling strategy

92 The study area is located in the River Bode catchment (Saxony-Anhalt, Germany) of the
93 TERENO area, a long−term observatory focused on investigating the effects of climate and land
94 use changes on regional scale.[19,20] In order to tackle our hypotheses, three streams were selected
95 based on their degree of land use. We chose a non−impacted reference stream (Ochsenbach
96 (OC)) and two anthropogenically impacted streams (Sauerbach (SB) and Getel (GE)). The OC is
97 located in the Harz National Park and has a forested catchment and low nutrient concentrations

whereas SB and GE are dominated by arable land and are affected by agriculture (Table S1). In addition, GE receives the discharge of a wastewater treatment plant (WWTP; mean population equivalent=6,833) located 4 km upstream from our sampling site resulting in both agriculture and urban inputs into this stream. The streams are only connected several kilometres downstream from the sampling sites; therefore, no organism exchange−based bias is expected in the selected sampling sites.

G. pulex was collected monthly by kick sampling (Surber sampler, 250 μm mesh) from September 2012 to November 2013. At each stream, five samples were taken distributed over a stretch of 50 metres, corresponding to a total sample area of 0.3125 m². Specimens were sorted alive and 30 individuals were stored in 95 % ethanol. A second set of individuals were stored at −20 °C for later body burden analysis of OMPs. In both cases, individuals representing the entire available body size range were collected in order to avoid biases produced by different ages of specimens.

Body burden and chemical analysis of OMPs

A total of 74 analytes with a wide range of hydrophobicity (log K_{OW} from −0.21 to 5.51) were selected for internal concentration analysis based on their occurrence in water samples and sediments.[21–24] The selected compounds belong to different classes of pollutants, such as pesticides, pharmaceuticals, industrial chemicals and some of their main transformation products (Table S2). The OMPs were extracted from *G. pulex* using a multi− and non−target screening method based on pulverised liquid extraction (PuLE) and a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method with an additional hexane phase.[9] More details on the sample preparation are given in the SI, section S1.1.

120 Toxicity of the OMP mixtures

121 The toxicity of environmental chemicals which aquatic organisms are exposed to can be
122 estimated using prediction models, such as the concentration addition (CA) model. In aquatic
123 toxicology the CA model is widely used for the assessment of combined effects and is based on
124 the toxicities of the individual compounds and their concentrations in the mixture.[25,26] Because
125 toxicity data is largely reported in dissolved concentrations, equilibrium partitioning theory was
126 applied to calculated their freely dissolved forms. Once freely dissolved water concentrations
127 (c^{fd}) were calculated, they were used for mixture toxicity predictions using the CA modelling
128 technique. More details on calculations of dissolved concentrations and CA modelling are given
129 in the SI, section S1.2.

130 Microsatellite analysis

131 DNA was extracted using a NucleoSpin® 96 Tissue Kit (Macherey−Nagel) following the
132 manufacturer's recommendations. Concentration and quality of DNA was measured using a
133 NanoDrop ND−1000 (NanoDrop Technologies Inc.). DNA was amplified by polymerase chain
134 reactions (PCRs) using nine microsatellite markers (Table S4). Five loci were amplified and
135 genotyped using primers previously developed for *G. pulex*.[27] Four additional markers were
136 originally designed for the sibling species *Gammarus fossarum*[28,29] which were also applied to *G.*
137 *pulex* in this study. More details on PCR configuration and fragment analysis are given in the SI,
138 section S1.3.

139 Genetic variation and differentiation

140 Genotypic data were analysed with MICRO−CHECKER 2.2.3[30] to detect and correct null alleles
141 and stutter peaks. The presence of F_{ST} outliers was evaluated using LOSITAN[31] by running

142 5×10^5 simulations with a confidence interval of 0.95. The genotype accumulative curve using the
143 R−package *poppr*[32] was constructed by randomly sampling *x* loci and counting the number of
144 observed multi locus genotypes. These curves are useful as statistical power analysis for
145 determining the minimum number of loci necessary to discriminate population structure with 95
146 % confidence.

147 Number of alleles (N_A), and the observed (H_O) and expected heterozygosity (H_E) were calculated
148 with Arlequin 3.5.[33] Exact test of Hardy−Weinberg equilibrium (HWE; 10^6 steps in the Markov
149 Chain Monte Carlo (MCMC) and 10^5 dememorization steps), and Linkage Disequilibrium (LD;
150 10^4 permutations) tests were performed with Arlequin 3.5. The False Discovery Rate (FDR)
151 approach was used to correct the results for multiple tests in both the HWE and LD. Genetic
152 differentiation was calculated based on both Wight's F_{ST},[34] which considers biallelic variation,
153 and G'_{ST},[35] which standardises G_{ST} to the maximum level of genetic variation
154 within−subpopulations, using the R−package *diveRsity*.[36] Allelic richness (A_R) and private alleles
155 (N_{PA}, i.e. alleles occurring in only one cluster) were calculated with HP-RARE 1.1[37] using
156 rarefaction to correct for differences in total number of alleles.

157 Finally, recent population bottlenecks were analysed using BOTTLENECK version 1.2.04.[38]
158 This software assumes that populations that have experienced a recent reduction in effective
159 population size (N_E) exhibit a more rapid reduction in allelic diversity than heterozygosity at
160 polymorphic loci. Hence, in a recently bottlenecked population, gene diversity is higher than the
161 equilibrium heterozygosity estimated from the observed allele numbers under the assumption of
162 mutation−drift equilibrium.[39,40] The Wilcoxon sign−rank test was performed to determine the
163 significance of heterozygosity excess, as recommended by Piry *et al*.[38] for analyses where the
164 number of polymorphic loci is small. We used two phase model (TPM) and stepwise mutation

model (SMM), default settings and combinations of 95% single-step mutations and 5% multistep mutations as recommended by Piry *et al.*[38] throughout the analyses.

Population structure

The presence of population clusters was assessed using STRUCTURE 2.3.4,[41] assuming an admixture model and correlated allele frequencies with LOCPRIOR turned off. STRUCTURE was run for $K = 1$ to $K = n+1$, where n is the maximum number of sites sampled in each stream system (i.e. OC, SB and GE). Ten independent runs were conducted for each K, with 2×10^5 burn−in periods, followed by 1×10^6 MCMC steps for each site. The likelihood results were collected and assessed in STRUCTURE HARVEST.[42] The Evanno method[43] was used to detect the number of clusters. The Greedy algorithm in CLUMPP 1.1.2[44] was used to create a single plot based on the ten independent runs, and the final graphic results were obtained with DISTRUCT 1.1.[45]

Theta (θ), a population mutation parameter that measures the capability of a population to maintain genetic variability,[46] was calculated using MIGRATE−n.[47,48] Our estimations used a Brownian motion model and included 1 long chain and 5 replicates. The parameter θ followed an exponential distribution. Burn−in was set at 5×10^5, with a sampling increment of 50 and a total of 5×10^4 recorded steps. Heating was set at 4 temperatures (1.0, 1.5, 3.0 and 10^6) using a static scheme. All other parameters were set to default. MIGRATE−n does not allow the assumption of null migration between two populations, therefore migration=0.01 was assumed to represent absence of migration following the author´s recommendations. The four models were compared using the Bezier implementation from MIGRATE−n based on the log marginal likelihood (lmL)

of the posterior probabilities to detect the best fit model following the methods of Beerli & Palczewski.[49]

Statistical analyses

We determined the trend and direction of significant inflections for population genetic patterns, such as genetic diversity (A_R), genetic differentiation (F_{ST}) and theta (θ) using generalised additive modelling (GAM) to relate variability of the response variables to anthropogenic and natural drivers. The anthropogenic driver was the toxicity from OMPs whereas discharge was used as a natural driver, indicating hydrological variability. In fact, freshwater invertebrates[50] and fish[51] have both shown alterations in their genetic diversity and genetic structure respectively due to discharge.

To test for the influence of toxicity and discharge on A_R, F_{ST} and, θ we used the function "gam" in the R−package *mgcv*.[52] Each of the independent variables were linked to the categorical fixed factor stream and the variables "toxic unit" (TU) and discharge (Q) were fitted for each of the three streams as smooth terms using the "by" command based on the default method "tp" (thin plate regression splines). To account for temporal variation, interactions between streams and TU and/or Q were fitted using smooth terms with the "by" command. To test the hypothesis that temporal dynamics of A_R, F_{ST} and, θ differed between land use types, we used land use types as contrasts. A significant smooth term between the interactions sampling date and stream (SB or GE) indicated an observable difference from the temporal pattern of the reference stream (OC). A correlation structure ("Ar.start"), accounting for temporal autocorrelation was added to the model. This assigned each individual time series a start value using the "start_event" function and calculated the autocorrelation value of data points at lag 1 of the model without the

208 correlation structure using "start_value_rho" in the R−package *itsadug*.[53] Models for A_R and F_{ST}
209 were calculated based on gamma distributions using a log link function and the model for θ
210 based on a Gaussian distribution with a log link function. The function "gam.check" was
211 employed to visually evaluate the normalised residuals of the fitted models in regard of
212 normality of errors and constant variance.

213 To test for significant differences in A_R, F_{ST} and, θ and TU between the three streams,
214 generalised linear mixed models (GLMM)[54] were applied using the "glmer" function from the
215 R−package *lme4*, as there is no option to carry out pairwise comparisons between levels of a
216 factor based on GAM models. The model contained the fixed factor stream and a random slope
217 term, allowing the temporal variable sampling time to vary by the stream. Subsequently pairwise
218 comparisons were conducted using the function "lsmeans" in the R−package *lsmeans*. In order to
219 avoid collinearity between predictor variables, we carried out Pearson correlations and found no
220 variable to have a correlation coefficient $r > 0.3$.

221 Results

222 OMP concentrations

223 We quantified a total of 26 out 74 pollutants in *G. pulex* extracts and we observed distinct
224 internal OMPs profiles between the studied streams supporting our first working hypothesis
225 (Figure 1). In general, *G. pulex* organisms inhabiting the anthropogenically impacted streams
226 (SB & GE) revealed a significantly higher number, concentration, and toxicity of OMPs
227 compared to the reference stream (OC).

228 Similarly, the streams affected by anthropogenic activity (SB and GE) showed a high number
229 and concentration of OMPs in gammarid extracts (Figure 1), and also a higher frequency of the

insecticides imidacloprid and thiacloprid, the fungicide spiroxamine, the herbicide diflufenican and the wastewater−derived chemical 5MBT (Table S5 and S6). Moreover, the herbicide fenuron together with the wastewater−derived chemicals carbamazepine, CBZ−diol and benzotriazole were frequently quantified in GE (Table S6). Overall, the stream GE, impacted additionally by both agricultural run−off and WWTP discharge, showed the highest number of OMPs in *G. pulex* extracts ($p < 0.001$) relative to both the agriculturally affected (SB) and reference stream (OC). Although OMPs were not expected in the reference stream (OC), the herbicide fenuron was quantified with high frequency (Table S7). Most importantly, the lack of overlap of OMP's in the reference stream with the anthropogenically impacted streams reinforces our assumption that there was no population migration between the sampling localities.

In general, the anthropogenically impacted streams had the highest toxicity values compared to the reference stream ($p < 0.001$). Indeed, the highest toxicity values were calculated in GE ($p < 0.001$). Particularly, the insecticide imidacloprid dominated the toxicity profiles reaching the highest TU values. Overall, distinct OMP internal profiles were supported by specific chemicals, gradient concentrations and mixture toxicity patterns in different land use stream ecosystems.

Genetic variation and bottlenecking

The genotype accumulative analysis determined an asymptote and a decrease in variance in eight microsatellite loci with a 95% confidence in the sampled populations (Figure S1); additionally, no outliers were detected in any of the nine loci (Figure S2). Although specific locus departure from HWE occurred, no global population deviations from HWE were detected and no evidence of LD was observed after applying FDR correction for multiple tests. Wright's *F*−statistics, especially the inbreeding coefficient (F_{IS}), were negative in the reference stream OC (Table S8).

252 However, 3 out of 15 and 5 out of 15 months were positive with values very close to zero in SB
253 and GE, respectively. These positive values were more common in GE than in SB. The lowest
254 F_{IS} was measured in OC (October 2012, $F_{IS} = -0.53$) and the highest F_{IS} were detected in the
255 impacted streams, December 2012 in SB and June 2013 in GE ($F_{IS} = 0.08$; Table S8).

256 We calculated general population genetic metrics for each sampling campaign and by stream
257 using the following criteria: theta (θ), allelic richness (A_R), observed heterozygosity (H_O),
258 expected heterozygosity (H_E), and number of private alleles (N_{PA}). The results are summarised in
259 Table S8. GE showed the highest values for several genetic parameters such as A_R, H_E, and N_{PA}
260 and conversely, the reference stream OC showed the lowest values (Table S8). However, both
261 monthly and total period metrics were not significantly different between the reference and
262 impacted streams, with the exception of A_R.

263 Allelic richness (A_R) was significantly higher in the anthropogenically impacted streams than in
264 the reference stream ($p < 0.001$, Figure 2) and A_R between the two impacted sites did not
265 significantly differ (Fig. 2). Additionally, gammarids in OC showed fewer fluctuations in A_R
266 with a standard deviation of 0.13 compared to 0.29 and 0.33 in SB and GE respectively (Figure
267 S3). Declines in genetic diversity were only identified and associated with higher discharge rates
268 in anthropogenically impacted streams (e.g., SB and GE; Figure S3), suggesting further
269 implications in other population genetics parameters. The total number of alleles and allelic
270 frequencies were plotted for OC, SB and GE (Figure S4, S5 and S6 respectively). We observed
271 important changes in allele frequencies in the same months where STRUCTURE showed
272 changes in the genetic structure of *G. pulex*. However, these changes in alleles did not lead to
273 any observed modification in the number of alleles.

Populations differed in the extent of their genetic differentiation ($G`_{ST}$ and F_{ST}) with an increase of differentiation measured from the reference to the anthropogenically impacted streams. In OC, the highest level of differentiation was detected in January 2013 and to a lesser extent in November 2012 showing a certain degree of differentiation with respect to September and October 2012 (Figure S7). Regarding the anthropogenically impacted, agricultural stream SB showed remarkable changes along the monitoring period in its pairwise−differentiation in September 2012, January 2013 and April 2013 (Figure S8). Meanwhile GE, which is additionally under the pressure of a WWTP, exhibited high degrees of genetic differentiation over the sampling period (September 2012, December 2012, January 2013 and May 2013) (Figure S9).

Statistically significant bottlenecking occurred during the monitored period in each stream. The non−parametric Wilcoxon rank test revealed bottlenecks in October 2012 in OC, in September 2012 and June 2013 in SB, and in June 2013 in GE (Table S9). However, certain biases were observed in the calculated recent bottlenecking analysis in OC, where heterozygosity excess was influenced by the lower number of individuals collected in October 2012, which would have impacted heterozygosity metrics in this month.

Population Structure

Changes in the genetic structure were documented between and within streams associated with anthropogenic activity. STRUCTURE revealed only two clusters (K = 2) between stream analysis along the monitoring period. The reference stream (OC) was clearly distinguished from the impacted streams affected by agricultural and WWTP activities (Figure 3A). Within each stream, STRUCTURE showed two clusters for OC (K = 2) and five clusters for both SB and GE (K = 5) (Figure 3B). For OC, one cluster was September−October 2012 and the second was the

296 rest of the monitoring period. In the case of SB, the five clusters were September 2012,
297 December 2012, January 2013, April 2013 and the rest of the monitoring period. Finally, for GE,
298 clusters were identified in September 2012, December 2012, January 2013, May 2013 and the
299 rest of the monitoring period. Therefore, the main changes in *G. pulex* were identified during the
300 fall and winter seasons in all streams and in April 2013 and May 2013 in SB and GE,
301 respectively (Figure 3B).

302 Subsequently intra-stream analyses were conducted in order to investigate more complex
303 structuring per stream. STRUCTURE showed two clusters for November 2012 and January 2013
304 in OC (Figure S10A), and, in order to discard any masked cluster in winter, two extra runs were
305 performed adding to the analysis February 2013 (November 2012 - January 2013 – February
306 2013, Figure S10B) and March 2013 (November 2012 - January 2013 – February 2013 – March
307 2013, Figure S10C). The Evanno method revealed a $K = 3$ for the later runs showing independent
308 clusters in November 2012 and January 2013. No significant structure was found for the
309 September-October 2012 and November 2013 periods (Figure S10D). Finally, we investigated
310 any further substructure along with the rest of the genetic monitoring and Evanno gave a $K = 2$.
311 However, DISTRUCT did not find a significant population structure (Figure S10E).

312 In the stream principally affected by agricultural activity SB, the Evanno method revealed a $K =$
313 5 for the whole monitored period (Figure 3B). To confirm and/or unmask further clusters, further
314 analyses were carried out (Figure S11). STRUCTURE confirmed four changes in the genetic
315 structure (i.e., September 2012, December 2012, January 2012 and April 2013) of *G. pulex*
316 populations (Figure S11A). The decomposition of the observed clustering was conducted to rule
317 out any uncertainty about the clustering (see Figures S11B and S11C). Finally, an independent

subset of data spanning the period from May 2013 to November 2013 showed a $K = 2$. However, DISTRUCT did not find a significant population structure here (Figure S11D).

In the case of the stream influenced by agricultural and WWTP activities, the divergent months of September 2012, December 2012, January 2013 and May 2013 were analysed showing a $K = 2$ (Figure S12A). A second and third temporal analysis was performed for identifying new substructures among these months (see Figure S12B and S12C). STRUCTURE analysis identified independent structures for the previously mentioned months, confirming substructures for September 2012, December 2012, January 2013 and May 2013. Finally, an independent subset of data spanning from October 2012 to November 2013, excluding the mentioned months, revealed a $K = 2$ in GE. However, DISTRUCT did not find a significant population structure here (Figure S12D).

Relationship between stressors and population genetic metrics

Our analyses demonstrated that toxicity and discharge did not influence genetic differentiation (F_{ST}) in any of the streams (Table S10). However, significant changes in theta and A_R trends were determined for both toxicity and discharge variables and for their interaction (Table S10). Discharge had a significant effect on theta in both SB and GE (edf = 1, F = 6.596, $p = 0.0003$ and edf = 1, F = 0.430, $p = 0.0172$, respectively) as well as toxicity was significant on theta only in SB (edf = 1, F = 10.679, $p = 0.0033$). Nevertheless, the interaction of discharge and toxicity only significantly affected theta in SB (edf = 3, F = 8.295, $p = 0.0005$). Regarding A_R, discharge significantly affected diversity trends in SB and GE (edf = 1, F = 5.953, $p = 0.0220$ and edf = 2, F = 6.593, $p = 0.0057$, respectively) and toxicity affected only GE (edf = 1, F = 4.581, $p = 0.0421$). Furthermore, discharge and toxicity interaction influenced significantly A_R trends in SB

and GE (edf = 1, F = 4.431, p = 0.0453 and edf = 1, F = 9.352, p = 0.0051, respectively). Therefore, our stressor variables, discharge and toxicity, significantly affected streams influenced by agricultural and WWTP activities.

Discussion

In this study, we gained new insights into how emerging pressures such as chemical pollution and land use, drivers with wide spatial and temporal implications, can alter genetic patterns in freshwater invertebrate populations. Overall, our results indicate distinct internal OMP profiles and changes in both genetic variation and genetic structure in streams affected by anthropogenic activity. These outcomes were attributed to both chemical pollution (genetic variation) and changes in discharge (genetic structure) in anthropogenically impacted stream ecosystems working both independently and in tandem.

The use of internal concentrations of OMPs in freshwater macroinvertebrate extracts is in good agreement with the exposome concept suggested by Rappaport and Smith,[55] and lately reviewed by Escher *et al*.,[7] where the analysis of exogenous chemicals is tightly interlinked with adverse outcomes in exposed organisms. The OMPs measured in this study were higher than in other studies sampling gammarid populations[9] and specifically *G. pulex*.[4,8] These high internal concentrations may be explained by the role of discharge and how it may drive the final exposure concentration of OMPs. Our study focused on small streams with low discharges (e.g., mean annual flow in our streams: 31 ± 26 L/s), in contrast to previous studies conducted in river systems such as River Thames[8], Holtemme[4] and Danube[9] with much higher discharges and thus contaminant dilution compared to the streams in this study. Predicted toxicity profiles followed same distinct internal profiling, which is in line with predicted findings in the River Holtemme

where *G. pulex* populations were exposed to a pollution gradient along the river.[4] Nine out of the 26 quantified chemicals in *G. pulex* extracts have shown mutagenic features in both *in vitro* and/or *in vivo* testing (e.g., imidacloprid,[56–58] thiacloprid,[59] diazinon,[60] fipronil,[61] azoxystrobin,[62,63] pendimethalin,[64] metolachlor,[65] carbamazepine[66] and 1H-benzotriazole[67]). The presence of these OMPs have the potential to affect population genetic patterns, for instance, inducing *de novo* mutations and thus increasing mutation rates or selection of rare genotypes in highly impacted streams. Our results reveal a high presence of private/rare alleles for *G. pulex* in GE, the stream affected by agricultural and WWTP discharges. Private/rare alleles have been used as proxy for mutagenicity activities in polluted sites previously for fish and *G. pulex* populations.[4,68,69] Although the individual or collaborative effects of chemicals causing mutagenic effects are beyond the remit of this study, the high number of private/rare alleles in GE suggest a OMP from the WWTP and/or from the agricultural activities may potentially have such an effect. WWTPs and agricultural runoffs are known sources of chemicals with mutagenic features affecting aquatic organisms[60,70,71] and several studies have shown that chemical pollution affect the variability of microsatellites.[72–74] Hence, OMP-derived mutagenic effects resulting from anthropogenic land use cannot be ruled out. Finally, our internal concentration clearly reveal that streams associated with anthropogenic activities use result in higher OMP concentrations and distinct profiles relative to our control stream. Confirming our first hypothesis.

With respect to our second hypothesis, contrary to prediction, our results showed a higher genetic diversity, expressed as A_R, in streams under long−term exposure of OMPs. The significantly higher neutral genetic diversity in streams influenced by OMPs from agricultural and WWTP activities suggests novel mechanisms enhancing genetic diversity in these

anthropogenically impacted stream ecosystems. Several authors[75–77] have found that A_R more accurately reflects changes in the genetic composition of a population than heterozygosity, especially after short−term processes affecting the studied populations (decades, not hundreds or thousands of years). Therefore, our findings in A_R are consistent with recent changes in genetic variation in *G. pulex*. As mentioned earlier, a potential explanation to this high genetic diversity is the presence of pollutants with mutagenic features, which may interact with exposed *G. pulex* increasing its mutation rates and ultimately genetic diversity.[78,79] Nevertheless, we cannot neglect that the observed patterns could be the result of founder effects, where the reference stream OC had originally lower A_R than populations from SB and GE, even before changes in the landscape occurred. However, if a long−term process for which founder effect is responsible for, it would be more likely to observe this pattern in the observed heterozygosity, not in the number of alleles as suggested by Maruyama & Fuerst.[75] Historical patterns could also be responsible for limiting genetic drift. Theoretically, genetic drift is linked to habitat size (e.g., habitat area, volume, patches, resources, etc) and effective population sizes. Thus, if habitat sizes and/or effective population sizes in SB and GE were larger originally in these populations than in OC, we could expect to have a smaller effect of drift in the impacted streams. As a result, lower A_R may be expected in OC due to limited habitat size and reduced effective population sizes.

Changes in genetic diversity may have direct implications on population fitness and therefore may alter ecosystem functioning.[80–82] In this line of reasoning, population bottlenecking, a neutral genetic process, is prone to cause losses of adaptive potential in affected populations.[83] Although our bottlenecking results are not conclusive, because our two mutation models (i.e., two−phase and stepwise mutation models) showed different results for recent bottlenecking, we observed significant recent bottlenecks in all streams in this study. The reference stream

experienced a bottleneck in October 2012 that was not associated with any stress parameter. However, we observed bottlenecking in June 2013 in SB and GE only after highest flood events. Indeed, this is much in line with reports identifying flood events as responsible of causing severe bottlenecks and a subsequent promotion of genetic diversity in snail populations.[50] Our results support the driving role of discharge on genetic diversity and furthermore, we extend this driving function, based on our modelling results, when discharge interacted with chemical pollution. Recent investigations reported changes in population genetics patterns due to the independent action of chemical pollution[5,84] and discharge.[85] Nevertheless, to the best of our knowledge, this is the first study reporting the tandem effects of the aforementioned stressors in long−term exposed freshwater invertebrate populations from a genetic perspective.

When assessing the capability of *G. pulex* populations to maintain their genetic variability, theta parameter (θ), we observed that populations living in anthropogenically impacted streams (SB and GE) exhibited lower capabilities to maintain their genetic variability and additionally they were more sensitive to both natural and anthropogenic stressors from a structural perspective. Using both similar and dissimilar mutation rates (μ) between the studied populations (i.e. dissimilar due to the action of mutagenic pollutants) had no significant impact; instead large effective population sizes (*N*e) were the principal factor affecting θ in both scenarios. Therefore, the effect of genetic drift may be discarded and especially on low A_R populations (for instance the reference site OC). This leads us to suggest that the higher A_R in SB and GE could be the result of chemical pollution.

Regarding our final hypothesis, our results confirmed that long−term exposure to OMPs result in more complex genetic structures than populations living in non−impacted stream ecosystems. We hypothesised that these structural changes are associated to both environmental disturbances

and the effect of chemical pollution. In fact, environmental disturbances have been suggested as having a shaping role for neutral genetic processes such as genetic structure as well as selective processes on natural populations.[86] Disturbances may reduce genetic diversity within populations (in particular, allelic diversity or richness), when population sizes are reduced. Reductions of A_R are anticipated, where mortality is high and population recovery stems from a few *in situ* survivors, and where ongoing migration is low relative to population growth rate.[86] In particular, flood events exerted severe changes in the genetic structure of *G. pulex* populations in spring. These changes were only pronounced in anthropogenically impacted streams. That is, the presence of OMPs may cause direct and indirect effects on population genetics parameters,[87,88] for instance enhancing private/rare alleles in SB and GE, and these "new" genotypes may play a role taking over sensitive genotypes removed after floods. Similar structuring patterns and enhanced mutagenic activity due to high discharge have been reported in wild fish populations.[51,60,69,70] However, so far these studies have dealt with these stressors independently, and here we clearly demonstrate how adverse outcomes of both stressors can act simultaneously in invertebrate freshwater populations.

We conclude that streams associated with anthropogenic activities are prone to suffer remarkable changes in their population genetic patterns compared to non−impacted stream ecosystems. Clear distinct pollution profiles, high allelic diversities and complex genetic structures characterised streams with high load of pesticides and wastewater−derived pollutants. This study stimulates the new question whether similar patterns can also be observed at the community level in exposed freshwater invertebrate organisms. Moreover, in order to obtain new insights into further implications at functional level as well as to elucidate the mechanisms behind our

findings, complementary transcriptome studies are suggested in aquatic invertebrates exposed to complex chemical mixtures.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detail information about sample preparation, calculations of dissolved concentrations, and CA modelling, PCR setup and fragment analysis are described in Supporting Information. Figures showing genotype accumulative curves, outlier analysis, allelic richness, streamflows, allelic frequencies, genetic differentiation, and population genetic structure are given in Supporting Information. Tables showing physical-chemical features of the studied streams, chemical features of the targeted organic chemicals, ecotoxicity information, primer details, body burden in *G. pulex*, population genetic parameters, bottleneck information, and GAM outputs.

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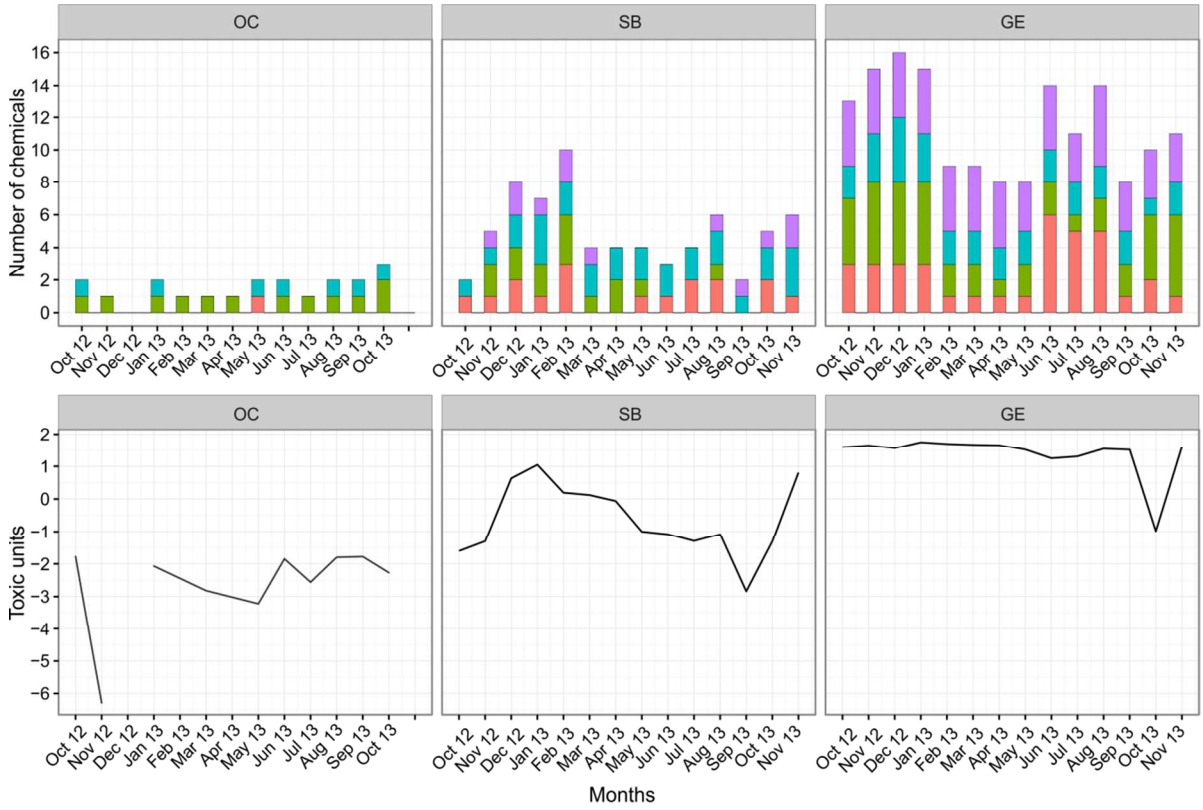

Figure 1: Number of chemicals quantified in *G. pulex* extracts (upper panel) and toxic pressure represented as toxic units (lower panel) in Ochsenbach (OC, non−impacted reference), Sauerbach (SB, agricultural) and Getel (GE, agricultural+WWTP). Chemicals are categorised into fungicides (pink), herbicides (green), insecticides (light blue) and wastewater-derived chemicals (purple).

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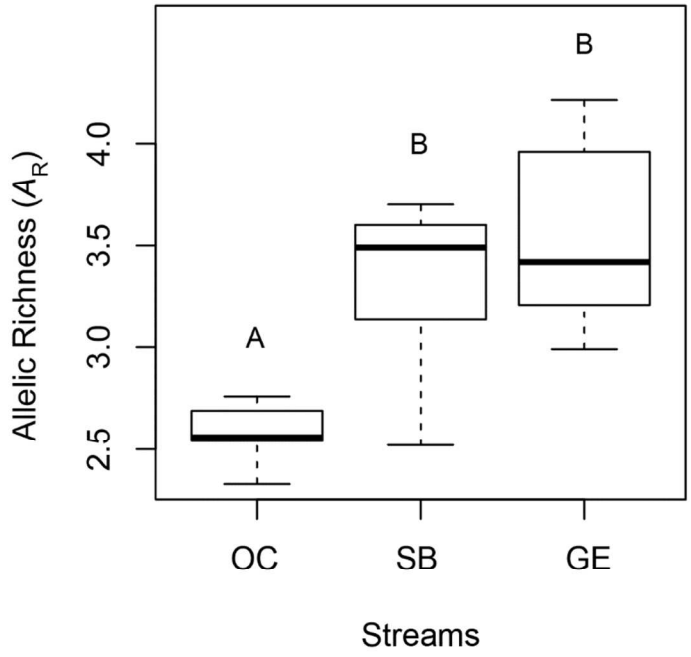

Figure 2: Genetic diversity of *G. pulex* represented as allelic richness (A_R) in the Ochsenbach (OC) and Sauerbach (SB) and Getel (GE). Significant differences are indicated by different letters (using $p<0.001$ as a threshold, Tukey`s post-hoc). Boxes delineate the 25 and 75% percentile, whiskers delineate the minimum and maximum A_R.

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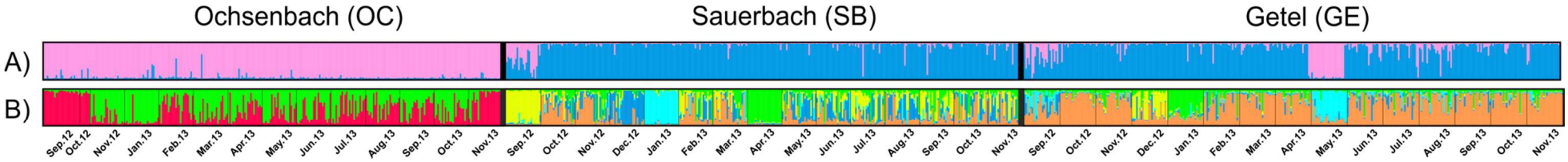

Figure 3: Population genetic structure of the 1027 *G. pulex* individuals from the non-impacted stream Ochsenbach and the impacted streams Sauerbach and Getel. A) Clustering of genetic structure into a reference and an impacted group (STRUCTURE analysis, Evanno`s method $K = 2$). B) Independent analyses per stream were conducted and clustering of the genetic structure into 2 groups for Ochsenbach and 5 groups for both Sauerbach and Getel (Evanno`s method $K = 2$ and $K = 5$ respectively). Each individual's genotype is represented by a thin vertical line, which is partitioned into coloured sections, which represent the different clusters.

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