

Supplementary Material

Nitrogen pollution in rivers as potential driver of invertebrate species turnover

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Part 1: Water parameters of sampling sites

Table S1: Water parameters measured at the three sampling sites / tributaries of the Nidda river in central Germany in autumn (October 2023) and winter (January/February 2024). Individuals from *G. fossarum*, *G. pulex*, and *G. roeselii* were collected from Urselbach, Westerbach and Horloff, respectively.

	URSELBACH		WESTERBACH		HORLOFF	
Season	Autumn	Winter	Autumn	Winter	Autumn	Winter
Temperature [°C]	10	7.4	11.2	6.4	10.8	6.8
Conductivity [µS/m]	91.6	103	312	184.5	563	489
pH	8.72	7.31	8.31	7	7.3	7.18
O2 [%]	95.8	93.7	90.2	95.6	77.2	83
NO ₂ -N [mg/l]	0.006	0.009	0.013	0.047	0.027	0.039
NH ₄ ⁺ [mg/l]	0.01	0.03	0.01	0.244	0.083	0.140
Carbonate hardness [°d]	1	0.8	4.5	1.8	11	7.8
Total hardness [°d]	<3	4.2	>6	3.6	13.6	11.4

Part 2: Acute nitrite tolerance

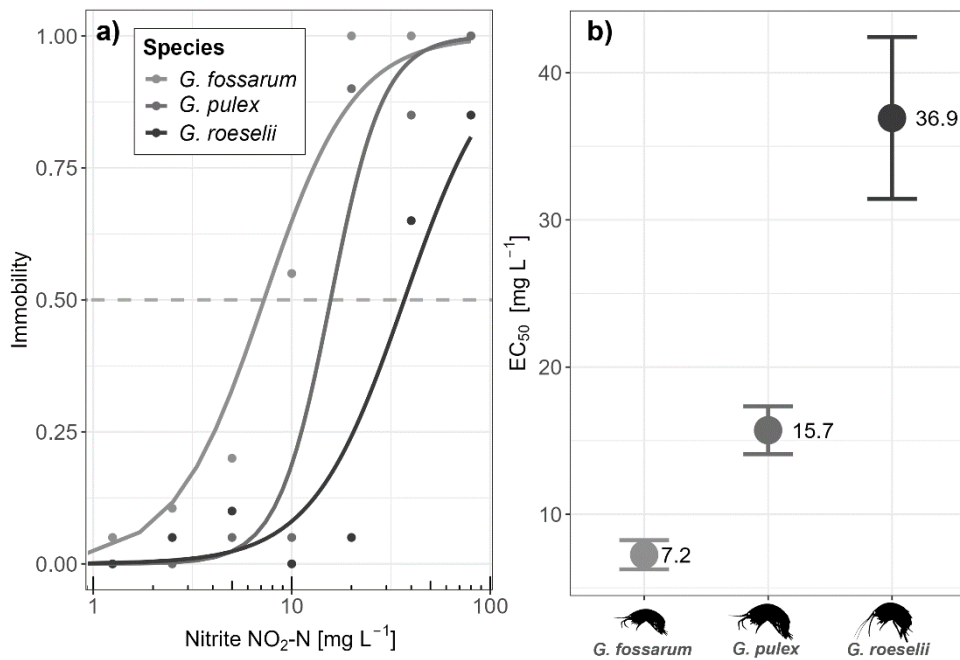


Fig. S1: (a) Concentration–response relationship of nitrite after 96 h for *Gammarus fossarum*, *G. pulex* and *G. roeselii*. Model is a two-parameter log-logistic function fitted using the drc-package in R. (b) shows the estimated effect concentration EC₅₀ for 96 h (± SE) calculated from the left panel data. The EC₅₀ values differed significantly among species, as indicated by pairwise comparisons shown in Supplementary Table S2.

Table S2: Estimated (± Standard error and 95% CI) EC₅₀ and LC₅₀ values at 24, 48, 72 and 96h. All values are reported in mg L⁻¹ N-NO₂.

SPECIES	TIME (H)	RESPONSE	ESTIMATE	SE	LOWER 95% CI	UPPER 95% CI
<i>G. fossarum</i>	24	EC ₅₀	14.7	1.59	11.54	17.76
<i>G. pulex</i>	24	EC ₅₀	32.2	4.24	23.92	40.53
<i>G. roeselii</i>	24	EC ₅₀	51.9	8.92	34.38	69.34
<i>G. fossarum</i>	48	EC ₅₀	11.5	1.34	8.86	14.11
<i>G. pulex</i>	48	EC ₅₀	20.9	2.55	15.84	25.85
<i>G. roeselii</i>	48	EC ₅₀	49.6	8.32	33.26	65.86
<i>G. fossarum</i>	72	EC ₅₀	9.99	1.11	7.81	12.18
<i>G. pulex</i>	72	EC ₅₀	16.9	1.96	13.01	20.71
<i>G. roeselii</i>	72	EC ₅₀	42.0	6.70	28.92	55.17
<i>G. fossarum</i>	96	EC ₅₀	7.55	0.90	5.78	9.32
<i>G. pulex</i>	96	EC ₅₀	14.7	1.58	11.54	17.76
<i>G. roeselii</i>	96	EC ₅₀	36.9	5.50	26.15	47.70
<i>G. fossarum</i>	24	LC ₅₀	21.5	2.32	16.92	26.03
<i>G. pulex</i>	24	LC ₅₀	67.7	10.8	46.51	88.86
<i>G. roeselii</i>	24	LC ₅₀	90.9	25.1	41.74	140.03
<i>G. fossarum</i>	48	LC ₅₀	19.5	2.43	14.68	24.22
<i>G. pulex</i>	48	LC ₅₀	35.7	5.38	25.16	46.24
<i>G. roeselii</i>	48	LC ₅₀	59.4	9.89	39.98	78.75
<i>G. fossarum</i>	72	LC ₅₀	17.5	2.16	13.26	21.72
<i>G. pulex</i>	72	LC ₅₀	26.3	3.70	19.00	33.52
<i>G. roeselii</i>	72	LC ₅₀	52.3	8.25	36.12	68.46
<i>G. fossarum</i>	96	LC ₅₀	16.3	1.98	12.41	20.18
<i>G. pulex</i>	96	LC ₅₀	19.6	2.62	14.43	24.70
<i>G. roeselii</i>	96	LC ₅₀	52.3	8.25	36.12	68.46

Table S3: Statistical output for EC₅₀ log-logistic models. Pairwise comparisons of the estimated model parameters (e-values) among the three species were conducted using Wald-type tests derived from the fitted log-logistic concentration–response model. Significant differences at $\alpha = 0.05$ between species are denoted by **bold** characters, indicating variations in sensitivity to nitrite among the tested species.

time		<i>G. fossarum</i> / <i>G. pulex</i>	<i>G. fossarum</i> / <i>G. roeselii</i>	<i>G. pulex</i> / <i>G. roeselii</i>
24	Estimate	0.455	0.282	0.621
	Std. Error	0.077	0.057	0.135
	t-value	-7.04	-12.5	-2.81
	p-value	< 0.001	< 0.001	0.005
48	Estimate	0.551	0.232	0.421
	SE	0.093	0.047	0.087
	t-value	-4.82	-16.2	-6.63
	p-value	< 0.001	< 0.001	< 0.001
72	Estimate	0.593	0.238	0.401
	SE	0.096	0.046	0.079
	t-value	-4.26	-16.5	-7.57
	p-value	< 0.001	< 0.001	< 0.001
96	Estimate	0.515	0.205	0.397
	SE	0.083	0.039	0.073
	t-value	-5.83	-20.4	-8.26
	p-value	< 0.001	< 0.001	< 0.001

Table S4: Statistical output for LC₅₀ log-logistic models. Pairwise comparisons of the estimated model parameters (e-values) among the three species were conducted using Wald-type tests derived from the fitted log-logistic concentration–response model. Significant differences at $\alpha = 0.05$ between species are denoted by **bold** characters, indicating variations in sensitivity to nitrite among the tested species.

time		<i>G. fossarum</i> / <i>G. pulex</i>	<i>G. fossarum</i> / <i>G. roeselii</i>	<i>G. pulex</i> / <i>G. roeselii</i>
24	Estimate	0.317	0.236	0.745
	Std. Error	0.061	0.070	0.237
	t-value	-11.1	-10.9	-1.08
	p-value	< 0.001	< 0.001	0.282
48	Estimate	0.545	0.328	0.601
	SE	0.107	0.068	0.135
	t-value	-4.27	-9.85	-2.95
	p-value	< 0.001	< 0.001	0.003
72	Estimate	0.666	0.334	0.502
	SE	0.125	0.067	0.106
	t-value	-2.68	-9.93	-4.68
	p-value	0.007	< 0.001	< 0.001
96	Estimate	0.833	0.312	0.374
	SE	0.151	0.062	0.077
	t-value	-1.11	-11.1	-8.08
	p-value	0.268	< 0.001	< 0.001

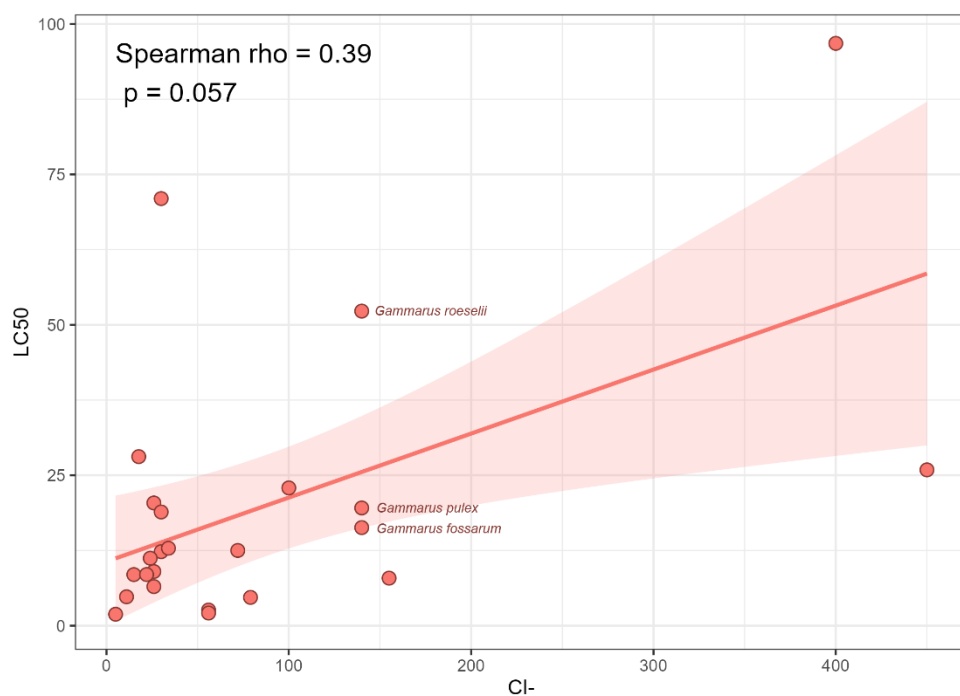


Fig. S2: Relationship between chloride content (Cl^-) and LC_{50} for Crustacea. Each point represents a study shown in Fig. 2. A Spearman correlation analysis showed $\rho = 0.39$, $p = 0.057$, indicating a slightly non-significant positive association between Cl^- and LC_{50} in Crustaceans. The three *Gammarus* species tested are labeled individually.

Part 3: Sub-lethal nitrite tolerance

2.1 Leaf consumption

Table S5: Number of individuals tested in the leaf consumption experiment and subsequently used for behavioral assessment in the Toxmate.

Treatment group	Individuals in leaf consumption exp. [n]	Individuals afterwards tested in behavioral exp. (Toxmate) [n]
<i>G. roeselii</i> Control	44	22
<i>G. roeselii</i> Nitrite	37	19
<i>G. fossarum</i> Control	46	23
<i>G. fossarum</i> Nitrite	46	23
<i>G. pulex</i> Control	56	28
<i>G. pulex</i> Nitrite	54	27

Table S6: Two-way Type III ANOVA results from linear model on leaf consumption rate.

Effect	Sum Sq	Df	F value	p
Species	0.125	2	60.9	< 0.001
Nitrite	0.036	1	35.0	< 0.001
Species × nitrite	0.021	2	10.4	< 0.001
Residuals	0.284	277		

Table S7: Pairwise comparisons of leaf consumption rates between species and nitrite exposure groups (NC = negative control; Nitrite = exposure to 1.0 mg L⁻¹ NO₂-N). Significant differences at α = 0.05 are highlighted in bold.

Contrast	Estimate	SE	df	t.ratio	p
<i>G. fossarum</i> NC - <i>G. pulex</i> NC	0.070	0.006	277	11.0	< 0.001
<i>G. fossarum</i> NC - <i>G. roeselii</i> NC	0.042	0.007	277	6.27	< 0.001
<i>G. fossarum</i> NC - <i>G. fossarum</i> Nitrite	0.040	0.007	277	5.92	< 0.001
<i>G. fossarum</i> NC - <i>G. pulex</i> Nitrite	0.074	0.006	277	11.5	< 0.001
<i>G. fossarum</i> NC - <i>G. roeselii</i> Nitrite	0.043	0.007	277	6.14	< 0.001
<i>G. pulex</i> NC - <i>G. roeselii</i> NC	-0.028	0.006	277	-4.32	< 0.001
<i>G. pulex</i> NC - <i>G. fossarum</i> Nitrite	-0.031	0.006	277	-4.82	< 0.001
<i>G. pulex</i> NC - <i>G. pulex</i> Nitrite	0.004	0.006	277	0.590	0.992
<i>G. pulex</i> NC - <i>G. roeselii</i> Nitrite	-0.027	0.007	277	-3.95	0.001
<i>G. roeselii</i> NC - <i>G. fossarum</i> Nitrite	-0.003	0.007	277	-0.418	0.998
<i>G. roeselii</i> NC - <i>G. pulex</i> Nitrite	0.031	0.007	277	4.84	< 0.001
<i>G. roeselii</i> NC - <i>G. roeselii</i> Nitrite	0.001	0.007	277	0.151	1.000
<i>G. fossarum</i> Nitrite - <i>G. pulex</i> Nitrite	0.034	0.006	277	5.34	< 0.001
<i>G. fossarum</i> Nitrite - <i>G. roeselii</i> Nitrite	0.004	0.007	277	0.552	0.994
<i>G. pulex</i> Nitrite - <i>G. roeselii</i> Nitrite	-0.030	0.007	277	-4.45	< 0.001

2.2: Behavioral response

Table S8: Mean $\pm$ standard deviation data of the behavioral parameters 'distance moved' and 'time active'.

Species	Treatment	Distance moved [m h ⁻¹]	Time active [%]
<i>G. fossarum</i>	Control	28.7 $\pm$ 17.9	77.7 $\pm$ 29.4
<i>G. pulex</i>	Control	19.2 $\pm$ 10.1	80.5 $\pm$ 26.3
<i>G. roeselii</i>	Control	12.0 $\pm$ 5.8	60.2 $\pm$ 22.1
<i>G. fossarum</i>	Nitrite	12.5 $\pm$ 9.4	56.1 $\pm$ 30.4
<i>G. pulex</i>	Nitrite	14.0 $\pm$ 13.9	59.0 $\pm$ 28.3
<i>G. roeselii</i>	Nitrite	12.3 $\pm$ 9.7	57.0 $\pm$ 27.4

Table S9: Statistical output of the analysis of deviance for the GLM examining the effects of species, nitrite, and their interaction on distance moved (TDM).

Effect	χ^2	Df	p
Species	16.1	2	< 0.001
Nitrite	14.8	1	< 0.001
Species $\times$ nitrite	7.81	2	0.020

Table S10: Pairwise comparisons of 'distance moved' between species and nitrite exposure groups (NC = negative control; Nitrite = exposure to 1.0 mg L⁻¹ NO₂-N). Significant differences at α = 0.05 are highlighted in bold.

Contrast	Estimate	SE	df	t.ratio	p
<i>G. fossarum</i> NC - <i>G. pulex</i> NC	0.403	0.202	136	1.99	0.352
<i>G. fossarum</i> NC - <i>G. roeselii</i> NC	0.870	0.214	136	4.06	0.001
<i>G. fossarum</i> NC - <i>G. fossarum</i> Nitrite	0.827	0.212	136	3.90	0.002
<i>G. fossarum</i> NC - <i>G. pulex</i> Nitrite	0.720	0.204	136	3.53	0.007
<i>G. fossarum</i> NC - <i>G. roeselii</i> Nitrite	0.843	0.223	136	3.78	0.003
<i>G. pulex</i> NC - <i>G. roeselii</i> NC	0.467	0.205	136	2.28	0.208
<i>G. pulex</i> NC - <i>G. fossarum</i> Nitrite	0.424	0.202	136	2.10	0.295
<i>G. pulex</i> NC - <i>G. pulex</i> Nitrite	0.317	0.194	136	1.64	0.577
<i>G. pulex</i> NC - <i>G. roeselii</i> Nitrite	0.440	0.214	136	2.06	0.315
<i>G. roeselii</i> NC - <i>G. fossarum</i> Nitrite	-0.043	0.214	136	-0.202	1.00
<i>G. roeselii</i> NC - <i>G. pulex</i> Nitrite	-0.150	0.206	136	-0.729	0.978
<i>G. roeselii</i> NC - <i>G. roeselii</i> Nitrite	-0.028	0.225	136	-0.123	1.00
<i>G. fossarum</i> Nitrite - <i>G. pulex</i> Nitrite	-0.107	0.204	136	-0.525	0.995
<i>G. fossarum</i> Nitrite - <i>G. roeselii</i> Nitrite	0.016	0.223	136	0.071	1.00
<i>G. pulex</i> Nitrite - <i>G. roeselii</i> Nitrite	0.123	0.215	136	0.571	0.993

Table S11: Statistical output of the analysis of deviance for the beta regression GLM on 'time active [%]', examining the effects of species, nitrite, and their interaction.

Effect	χ^2	Df	p
Species	8.71	2	0.013
Nitrite	5.84	1	0.016
Species $\times$ nitrite	3.61	2	0.164

Table S12: Pairwise comparisons of 'time active [%]' between negative controls (NC) of different species. Significant differences at $\alpha = 0.05$ are highlighted in bold.

Contrast	Estimate	SE	z.ratio	p
<i>G. fossarum</i> NC - <i>G. pulex</i> NC	-0.039	0.056	-0.69	0.9828
<i>G. fossarum</i> NC - <i>G. roeselii</i> NC	0.148	0.070	2.11	0.2809
<i>G. pulex</i> NC - <i>G. roeselii</i> NC	0.187	0.066	2.85	0.0498

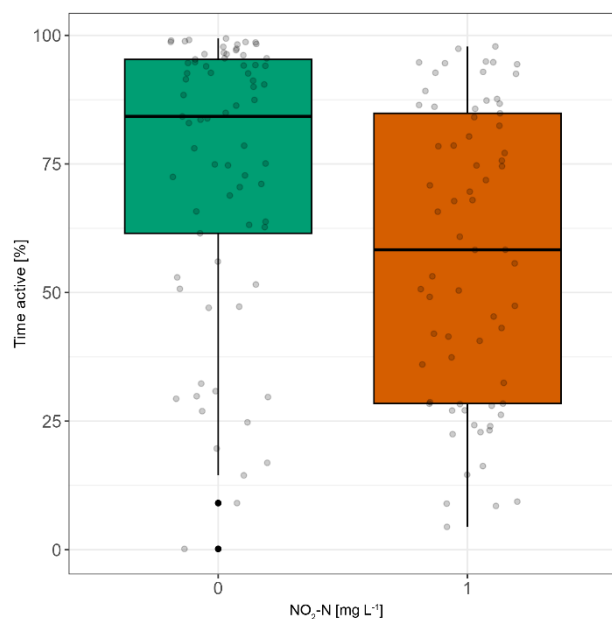


Fig. S3: Figure illustrates the significant impact of nitrite exposure on active time [%] across all species. After a 12-day exposure to 1 mg L⁻¹ nitrite, a clear reduction in activity was observed in the Toxmate. The negative control (no nitrite; green) provides a baseline for comparison, indicating the significant reduction of behavioral activity after nitrite exposure.