Wastewater alters feeding rate but not vitellogenin level of *Gammarus fossarum* (Amphipoda)

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Material and methods – complementary information

I) Reagents and chemicals

All chemicals were purchased from either AppliChem® (Darmstadt, Germany), Thermo Fisher Scientific Inc® (Waltham, USA) Honeywell Research Chemicals® (Bucharest, Romania), Merck® (Munich, Germany). Isotopically labeled ILIPGV*($^{13}C_5{}^{15}N$)GK peptide was purchased from Millegen® (Labège, France). Details are provided in Table S1.

II) Vitellogenin extraction followed by Liquid chromatography-mass spectrometry

The extraction was realized as described in Jubeaux et al. (2012). Briefly, *G. fossarum* were individually homogenized in 25 µL ice cold Tris buffer per mg wet body weight. Tris buffer contained 50 mM Tris, 100 mM NaCl and 0.01 mM EDTA (Ethylendiaminetetraacetic acid), the pH of the solution was adjusted to pH 7.8 followed by

spiking with 10 µg/L leupeptin and 10 µg/L aprotonin. Homogenates were centrifuged at 10.000 x g for 15 min at 4°C. Clear supernatant (250 µL) was taken out and delipidated by adding 750 µL of ethanol-diethylether solution (1/1, v/v). This mixture was vortexed and put on ice for 10 min. After the second centrifugation at 10.000 x g for 10 min at 4°C, all clear supernatant was removed and discarded. The pellet was mixed with 250 µL Tris buffer and quantitatively transferred into a 15 mL PP-centrifuge tube, 3 mL of 50 mM ammonium bicarbonate and 355 µL of 150 mM dithiothreitol were added to this PP-centrifuge tube. The mixture was incubated for 40 min at 60°C and allowed to cool down to room temperature before adding 395 µL 150 mM iodoacetamide. Afterwards, the tube was placed in the dark at room temperature for 40 min. Subsequently, 150 µL of the trypsin solution (2 mg/mL in 50 mM ammonium bicarbonate) were added and the samples incubated for 4 h at 37°C. The last steps were performed a second time by adding 455 µL of 150 mM dithiothreitol, followed by an incubation period of 40 min at 60°C, adding 505 µL of 150 mM iodoacetamide with a subsequent dark period of 40 min, adding 190 µL of 2 mg/mL trypsin solution and incubation for 20 h at 37°C. In the end 10 µL of formic acid (100%) was added to stop the reaction. To all samples 5 µL of ILIPGV*GK solution (10 ng/mL) was added as internal standard. ILIPGV*GK solutions were prepared independently in water-acetonitrile (95/5, v/v, acidified with 0.1% formic acid). The samples were vortexed, transferred to glass auto sampler vials for LC-MS/MS and stored at -20°C until further usage.

The samples were analyzed using a UPLC-MS/MS method (Xevo TQ, Waters, Milford, MA, USA). The Acquity UPLC System was used in combination with an UPLC BEH C_{18} Column (2.1x 50mm, 1.7 µm). 10 µL of the sample were injected into the column at 30°C. The mobile phase A was H_2O with 0.1% formic acid (v/v), the mobile phase B was Acetonitril with 0.1% formic acid (v/v). The samples were separated at a flow rate of 0.6 mL/min with a linear gradient from 5 to 15% B in the first 3 minutes, followed by 95% B until 5 minutes. Afterwards, the column was washed with 5% B for 2 minutes. Then the sample was positively ionized using ESI (electro spray ionisation) and, under vacuum, the

precursor ion was selected in the first quadrupole (m/z ratio: 398.8645). In the second quadrupole (called collision cell) the ions got aerated with Argon to divide into several product ions. In the third quadrupole these ions were analyzed for their m/z ratio again. ILIPGV*GK was used as internal standard. Valine (V) has 6 ^{12}C atoms which are changed to ^{13}C so that the mass is 6u higher.

III) Solid Phase Extraction of water samples for YES

Water samples were defrosted, filtered over 1 µm glassfibre filters (APFD 09050, Merck Millipore) with vacuum and adjusted to pH 3 with hydrochloric acid (HCl). Extraction was performed as described by Escher et al. (2008). Combined 6 mL EN/RP 18 cartridges (100 mg LiChrolut ethylvinylbenzene-divinylbenzene copolymer plus 250 mg LiChrolut RP-C18, Merck Millipore) were conditioned with 2 mL hexane, 2 mL acetone, 3x2 mL methanol and 3x2 mL nanopure water (pH=3). Afterwards, the water samples were enriched under vacuum through the columns. After extraction, columns were dried under a flow of nitrogen and eluted with 4x1 mL acetone and 1 mL methanol. Eluates were reduced to approximately 500 µL under a gentle flow of nitrogen and added up to 1 mL with ethanol.

IV) Passive sampling and extraction

For preparation and processing of the passive samplers, all equipment (beakers, forceps, steel holders and screws) was rinsed twice with acetone. Discs and membranes for the passive samplers were prepared as described in Vermeirssen et al. (2009): Empore SDB-RPS discs and PES membranes (both 47 mm diameter) were immersed slowly into methanol and shaken for 30 min on a plate shaker (MS 3 digital, IKA). Subsequently the methanol was discarded; discs and membranes were rinsed two times with nanopure water and afterwards shaken again for 30 min in nanopure water. Then, discs were covered with a membrane (with the shiny side up) and fitted into a stainless steel holder. The assembled samplers were kept in nanopure water until deployment (4°C). In the field,

two samplers per site were deployed for 7 days 100 m up- and downstream the WWTP effluent, respectively, in the middle of the river bed close to the *in situ* bioassays. After 7 days, samplers were removed and PES membranes were removed from the holder with forceps, coiled up and put carefully into brown 7 mL glass vials containing 7 mL methanol. The SDB discs were cut to the size of the membrane with a scalpel and put into a glass vial containing 7 mL acetone. All vials were closed, transported to the laboratory, and shaken for 30 min on a plate shaker. Subsequently, the solvent was transferred to another vial and reduced to 1 mL under a gentle flow of nitrogen. The glass vials containing the discs or membranes were filled again with 7 mL methanol, closed, and shaken for another 30 min. Afterwards, the solvent fractions were combined, and filtered with a glass syringe (PTFE Hydrophilic syringe filters, pore size 0.45 µm, BGB Analytik) before reducing the volume to below 1 mL and adjusting it to 1 mL with methanol.

V) Liquid Chromatography for chemical analysis

Chemical analysis was performed as described in Kern et al. (2009). In brief, 20 µL of each sample were injected into the device with an auto sampler (HTS PAL; CTC Analytics AG, Zwingen, Switzerland). A reversed phase column (XBridge™ C18 column, 3.5 µm, 2.1 x 50 mm; Waters, Ireland), a pre-column (same material, 2.1 x 10 mm) and a pump (Rheos 2200; Flux Instruments, Switzerland) were used. The mobile phases were water (A) and methanol (B) both acidified with 0.1% formic acid. The flow rate was 200 µL/min. The 29 min chromatographic run started with 90% A and 10% B, being linearly reduced to 50% each over 4 min, and afterwards to 5% A and 95% B by min 17. This mixture ratio was kept for 8 min. At 25 min, the solvent mixture was again switched to 90% A and 10% B and kept until 29 min.

VI) Yeast Estrogen Screen

For the Yeast Estrogen Screen (YES) (conducted according to Routledge and Sumpter (1996)) the growth medium was prepared using 45 mL Minimal-Medium, 5 ml glucose solution, 1.25 mL aspartic acid, 0.5 mL vitamin solution, 0.4 mL threonine solution and 125 µL cupric sulfate. One hundred twenty five µL of 10-fold concentrated recombinant yeast cells (*Saccharomyces cerevisiae*) were thawed from a frozen stock and added to the growth medium before incubation on an orbital shaker for 24 h at 30 °C (Heidolph Incubator 1000, incl. orbital shaker Heidoph 1010, Germany). On the test day, the sample extracts were pipetted in 96 well microtitre plates in a 1:2 dilution series with a range of eight concentrations and two replicates per sample. 17β-estradiol served as reference substance with eight concentrations being assessed in three replicates (initial concentration in the well: $1.25*10^{-9}$ M in ethanol). Pure ethanol served as blank tested in 16 replicates. After complete evaporation of the solvent, the wells were inoculated with 200 µL test medium (growth medium as described above amended with 0.5 mL CPRG (chlorophenolred-β-D-falactopyranoside)-solution as colorant and yeast). For that, the yeast cells from the 24 h culture were counted with a Neubauer improved cell chamber and a volume of up to 5 ml added to the medium to reach a final density of $4*10^{7}$ cells per 50 ml medium. The plates were put on a plate shaker for 2 min and incubated afterwards for 71 h. After shaking for 2 min and incubating for one more hour plates were read after 72 h at 540 nm (to measure the color change) and 620 nm (to measure cell density) with a plate reader (Synergy2, Biotek, Winooski, United States).

Table S1: List of reagents and chemicals used

Chemical	Supplier
Acetone, CH3COCH3, Reag. Ph Eur	Merck
Adenine, C5H5N5, ≥99%	Honeywell Research Chemicals
Ammonium bicarbonate, CH5NO3, ≥99.0%	Merck
Ammonium bicarbonate, CH5NO3, ≥99.0%	Merck
Ammonium sulfate, H8N2O4S, ≥99.0%	Honeywell Research Chemicals
Aprotinin from bovine lung	Merck
Biotin, C10H16N2O3S, ≥99.0%	Honeywell Research Chemicals
Calcium chloride dihydreate, CaCl2*2H2O, ≥99.0%	Merck
Chlorophenol Red-β-D-galactopyranoside (CPRG), C25H22Cl2O10S, >90%	Merck
Copper(II) sulfate, CuO4S, >99.0%	Merck
Cyroterone, $C_{22}H_{27}ClO_3$, >95 %	Alpha Chemistry
D-(+)-Glucose C6H12O6 ≥99 %	Merck
Diethylether, (C2H5)2O, Reag. Ph Eur	Merck
Diethylether, (C2H5)2O, Reag. Ph Eur	Merck
DL-Dithiothreitol (DTT), C4H10O2S2, ≥99.5%	Merck
DL-Dithiothreitol, C4H10O2S2, ≥99.5%	Merck
D-Pantotohenic acid Calcium salt, C9H16NO5*0.5Ca	Honeywell Research Chemicals
Ethanol, absolute for analsis, C2H5OH, Reag. Ph Eur	Merck
Ethylendiaminetetraacetic acid, C10H16N2O8, ≥99%	Merck
Ethylenediaminetetraacetic acid, disodium salt dihydrate, >99%	Thermo Fisher Scientific
Formic acid, CH2O2, 98-100%	Merck
Formic acid, CH2O2, 98-100%	Merck
Iodoacetamide, C2H4INO, BioUltra	Merck
Iodoacetamide, C2H4INO, BioUltra	Merck
Iron(III) sulfate hydrate, Fe2O12S3*xH2O, Fe 21-23%	Merck
L-Argininmonohydrochlorid, C6H15ClN4O2	Merck
L-Ascorbic acid, C6H8O6, 99%	Merck
Leupeptin hemisulfate salt, microbial, ≥90% (HPLC)	Merck
L-Gluamic acid, C5H9NO4, ≥99%	Merck
L-Histidine, C6H9N3O2, ≥99.0%	Honeywell Research Chemicals

Chemical	Supplier
L-Isoleucine, C6H13NO2, >98%	Merck
L-Leucine, C6H13NO2, ≥98%	Merck
L-Lysine monohydrochloride, C6H14N2O2*HCl, ≥98%	Merck
L-Methionine, C5H11NO2S, ≥98%	Merck
L-Phenylalanine, C9H11NO2, ≥99.0%	Honeywell Research Chemicals
L-Serine, non-animal source, C3H7NO3	Merck
L-Threonin, C4H9NO9	AppliChem
L-Tyrosine, C9H11NO3, ≥98%	Merck
L-Valine, non-animal source, C5H11NO2	Merck
Magnesium sulfate heptahydrate, MgO4S*7H2O, ≥99.0%	Merck
Magnesium sulfate, MgSO4, >98%	Merck
Methanol, Optima, LC/MS	Thermo Fisher Scientific
myo-Inositol, C6H12O6, 99%	Merck
n-Hexane, CH3(CH2)4CH3, Reag. Ph Eur	Merck
Potassium chloride, ClK, ≥99.0%	Merck
Potassium hydroxide, HKO, ≥85%	Merck
Potassium phosphate monobasic, KH2PO4, >99.5%	Merck
Pyridoxine hydrochloride, C8H11NO3*HCl, ≥99.0%	Honeywell Research Chemicals
Sodium bicarbonate, CHNaO3, 99.7-100.3%	Merck
Sodium chloride, ClNa, >99.5%	Merck
Sodium hydroxide, HNaO, ≥98%	Merck
Thiamine hydrochloride, C12H17ClN4OS*HCl	Merck
Trizma base, C4H11NO3, >99.8%	Merck
Trypsin, from porcine pancreas, lyophilized powder	Merck

Table S2: Results of the leaf decomposition by the gammarids during the *in situ* experiment. The relative consumption as reduction of leaf mass corrected for biological activity and weight of the gammarid per day (mg dry leaf material per mg dry weight of *G. fossarum* per day) and the percentage of the consumption at downstream locations relative to the control location 100m upstream (in brackets).

Date	**12. - 19.04.2012**	**26.04. - 03.05.2012**	**10. - 17.05.2012**	**24. - 31.05.2012**	**Mean**
100 m upstream	0.191	0.167	0.272	0.330	0.240
100 m downstream	0.161 (84%)	0.143 (86%)	0.230 (85%)	0.190 (57%)	0.181 (75%)
200 m downstream	0.094 (49%)	0.145 (87%)	0.144 (53%)	0.166 (50%)	0.137 (57%)
400 m downstream	0.147 (77%)	0.183 (110%)	0.224 (82%)	0.316 (96%)	0.217 (91%)

Table S3: Results of the micropollutant screening of water samples in ng/sampler (SDB+PES) after one week exposure 100 m up- and downstream the WWTP effluent (semi quantitative, uncertainty 50%, detection limit 1-10 ng/sampler (SDB+PES)).

Substance	CAS-No	Upstream (ng/sampler)	Downstream (ng/sampler)	Active ingredient group	Sup-group
2,6-Dichlorobenzamide	2008-58-4	2.8	2.4	pesticide	
4-Acetaminoantipyrine (=N-Acetyl-4-	83-15-8		28	pharmaceutical	analgesic
4-Formamidoantipyrine (4-FAA)	1672-58-8		15	pharmaceutical	analgesic
5-Methylbenzotriazole	136-85-6		140	anti-corrosion	
Acesulfame	55589-62-3		6.4	food additive	sweetener
Atenolol acid (Metoprolol acid)	56392-14-4		<2	pharmaceutical	beta-blocker
Atrazine	1912-24-9	3.6	68	pesticide	
Atrazine-desethyl	6190-65-4	7.4	7	pesticide	
Azoxystrobin	131860-33-8		<2	pesticide	
Benzophenone-3 (=2-Hydroxy-4-	131-57-7		11.2	personal care	UV-filter
Benzotriazole	95-14-7		200	anti-corrosion	
Benzoylecgonine	519-09-5	<2	<2	narcotic	
Bezafibrate	41859-67-0		<2	pharmaceutical	lipid regulator
Bicalutamide	90357-06-5		<2	pharmaceutical	anti-androgen
Candesartan	139481-59-7		17.2	pharmaceutical	
Carbamazepine	298-46-4		20	pharmaceutical	antiepileptic
Carbamazepine-10,11-dihydro-10,11-dihydroxy	58955-93-4		38	pharmaceutical	
Carbamazepine-10,11-epoxide	36507-30-9		26	pharmaceutical	antiepileptic
Carbendazim	10605-21-7	<2	2	biocide	
Cetirizine	83881-52-1		4.4	pharmaceutical	antihistaminic
Chloridazon	1698-60-8	12.8	5.2	pesticide	
Chloridazon-methyl-desphenyl	17254-80-7	4.4	3.4	pesticide	
Chlorotoluron	15545-48-9		9	pesticide	
Clindamycin	18323-44-9		<2	pharmaceutical	antibiotic
Caffeine	58-08-2	<2	<2	tracer	
Diazinon	333-41-5	7.4	12.4	pesticide	
Diclofenac	15307-86-5		32	pharmaceutical	anti-inflammatory

Substance	CAS-No	Upstream (ng/sampler)	Downstream (ng/sampler)	Active ingredient group	Sup-group
Difenoconazole	119446-68-3		15	pesticide	fungicide
Diflufenican	83164-33-4		<2	pesticide	
Diuron	330-54-1	<2	52	biocide	
Diuron-desdimethyl	2327-02-8		2	biocide	
EDDP (2-Ethylidene-1,5-dimethyl-3,3-	30223-73-5	<2	<2	narcotic	
Eprosartan	133040-01-4	<2	<2	pharmaceutical	
Ethofumesate	26225-79-6	8.8	12.2	pesticide	
Fipronil	120068-37-3		<2	pesticide	insecticide
Fluconazole	86386-73-4		<2	pharmaceutical	antifungal
Fludioxonil	131341-86-1		<2	pesticide	
Flufenacet	142459-58-3	<2	5.6	pesticide	
Galaxolidone	256393-37-0		150	personal care product	
Hydrochlorothiazide	58-93-5		44	pharmaceutical	diuretic
Imidacloprid	138261-41-3		<2	pesticide	
Irbesartan	138402-11-6		11	pharmaceutical	
Isoproturon	34123-59-6	<2	<2	pesticide	
Lamotrigine	84057-84-1		32	pharmaceutical	
Lenacil	2164-08-1	<2	<2	pesticide	herbicide
Lidocaine	137-58-6		3.4	pharmaceutical	anethetic
Mecoprop	93-65-2		5.6	pesticide	
Mefenamic acid	61-68-7		2.4	pharmaceutical	anti-inflammatory
Metamitron	41394-05-2	38	36	pesticide	
Metamitron-desamino	36993-94-9	4.6	4.8	pesticide	
Metformin	657-24-9		<2	pharmaceutical	anti-diabetic
Metoclopramide	7232-21-5	<2		pharmaceutical	antiemetic
Metolachlor	51218-45-2	16.6	11.6	pesticide	
Metolachlor-ESA	171118-09-5	6	6	pesticide	
Metolachlor-OXA	152019-73-3	<2	<2	pesticide	
Metoprolol	37350-58-6		<2	pharmaceutical	beta-blocker

Substance	CAS-No	Upstream (ng/sampler)	Downstream (ng/sampler)	Active ingredient group	Sup-group
Metsulfuron-methyl	74223-64-6	<2	2	pesticide	
Monuron	150-68-5		<2	pesticide	
Myclobutanil	88671-89-0		<2	pesticide	fungicide
Mycophenolic acid	24280-93-1		7.2	pharmaceutical	immunosuppressant
N,O-Didesvenlafaxine	135308-74-6		<2	pharmaceutical	antidepressant
Napropamide	15299-99-7	<2	<2	pesticide	
Naproxen	22204-53-1		5.4	pharmaceutical	anti-inflammatory
O-Desvenlafaxine	93413-62-8		3	pharmaceutical	antidepressant
Oxazepam	604-75-1		4.4	narcotic	sedative
Pethoxamide	106700-29-2		<2	pesticide	
Phenazone (Antipyrine)	60-80-0		<2	pharmaceutical	analgesic
Primidone	125-33-7		4.6	pharmaceutical	antiepileptic
Promethryn	7287-19-6		<2	pesticide	
Prosulfocarb	52888-80-9	10.6	14.8	pesticide	
Simazine	122-34-9	<2	<2	pesticide	
Sitagliptin	486460-32-6		16.2	pharmaceutical	antidiabetic
Sucralose	56038-13-2		50	food additive	sweetener
Sulfamethoxazole	723-46-6		11	pharmaceutical	antibiotic
Sulfapyridine	144-83-2		<2	pharmaceutical	antibiotic
Telmisartan	144701-48-4		22	pharmaceutical	
Terbutryn	886-50-0	<2	<2	biocide	
Terbuthylazine	5915-41-3	2.4	12.6	pesticide	
Terbuthylazine-desethyl	30125-63-4	<2	<2	pesticide	
Thiacloprid	111988-49-9	<2	<2	pesticide	insecticide
Tramadol	27203-92-5	<2	<2	pharmaceutical	analgesic
Triclosan	3380-34-5		94	biocide	
Trimethoprim	738-70-5		<2	pharmaceutical	analgesic
Valsartan	137862-53-4		<2	pharmaceutical	angiotensin II antagonist
Venlafaxine	93413-69-5		<2	pharmaceutical	antidepressant

Table S4: Yeast Estrogen Screen: 17ß-estradiol equivalent (EEQ) values (ng/sampler) of passive sampler extracts. n gives the number of technical replicates per sample. LOQ = limit of quantification

Week	Date	Location	n	Mean EEQ (ng/sampler) (± SD)	LOQ (ng/sampler)
1	12.4 – 19.4.2012	upstream	2	not detectable	0.0
		downstream	2	0.17 (±0.07)	
2	26.4 – 3.5.2012	upstream	2	0.04 (±0.02)	0.04
		downstream	2	0.08 (±0.04)	
3	10.5 – 17.5.2012	upstream	2	0.03 (±0.01)	0.03
		downstream	2	0.05 (±0.01)	
4	24.5 – 31.5.2012	upstream	2	0.03 (±0.001)	0.02
		downstream	2	0.09 (±0.07)	

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