Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 574536, 7 pages
doi:10.1155/2011/574536

Research Article

GamTox: A Low-Cost Multimetric Ecotoxicity Test with *Gammarus* spp. for *In* and *Ex Situ* Application

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Received 4 February 2011; Accepted 9 April 2011

Academic Editor: Michelle Bloor

Gammarus spp. represent an important taxon in running water ecosystems concerning both structural and functional aspects. *Gammarus* spp. are also part of several macrozoobenthos indices for assessing biological water quality. However, in ecotoxicological water quality assessment, this taxon has been used much less than *Daphnia* spp. A new user-friendly and low-cost test protocol for *Gammarus* spp. has been developed, constituting the "ecotoxicological module "of an integrated multimetric triad-based concept for water quality assessment. The GamTox test is based on several test parameters: behavior (especially locomotion and feeding) depicts rapid and sensitive early warning indicators, survival displays an indicator of severe acute stress, and biochemical biomarkers, esp. AChE inhibition, is a sensitive marker of neurotoxic xenobiotic stress. GamTox can be performed both *in situ* and *ex situ*, based either on visual or automatical recording.

1. Introduction

Gammarus spp. contain a few hundred freshwater, brackish, and marine species in the Northern hemisphere [1]. They stand for important keystone species in aquatic ecosystems, both functionally and structurally, due to their high abundance and biomass [2, 3]. As shredders and detritus feeders, gammarids participate in the detritus cycle and microbial loop [4]. Moreover, they show a wide foraging plasticity, being also herbivores, predators, and even cannibalists. This flexibility might contribute to their ecological success in colonizing and invading ecosystems [2, 5] next to their high mobility (migration, drift), as well as their high reproductive capacity with several broods per female and year and the high number of offspring combined with a relatively long longevity (1-2 yrs).

Gammarids are sensitive towards different types of aquatic pollution [6]. Compared to *Daphnia magna,* gammarids are often more sensitive to metals or different types of pesticides, such as neurotoxic substances and especially pyrethroids (Table 1). In 17 from 57 studies gammarids were much more sensitive than *Daphnia magna*. As toxicity studies are difficult to compare due to different exposure times and test designs, a safety factor of 2 was applied to exptrapolate from an exposure time of 48 h in the *Daphnia magna* studies to 96 h in the *Gammarus* spp. studies, as usually the LC_{50}s decrease with increasing exposure time, esp. during the first 48 h [7]. After application of a correction factor of 2 to the *Daphnia magna* studies, sensitivity shifted towards *Daphnia magna* in some cases. As gammarids spend extended periods of time in close contact with bottom sediments and in the water/sediment contact zone, they are standard test species in ecotoxicity testing in the USA and Europe for testing acute toxicity of sediments ((mortality within 96 h), OPPTS 850.1020 guideline) [8]. There are many ecotoxicological studies and several tests based on different measurement parameters have been developed so far, some of them have successfully been applied for both *in situ* and *ex situ* tests; however, all these isolated scientific efforts have never been collated and combined to one multimetric multilevel test, such as the proposal of GamTox as presented here.

Behavioral parameters in ecotoxicology studies are characterised by short response times, sensitivity, ecological relevance, and noninvasiveness, allowing for repeated measures and time-dependent data analysis [9, 10]. Changes in behavior may be used as important indicators for ecosystem health, because they rest on biochemical processes, but also reflect the fitness of the individual organism as well as potential consequences on the population level, such as

Table 1: Comparison of sensitivity of *Daphnia magna* and *Gammarus* spp. towards different types of chemicals. Daph-48: LC_{50}-48 h for *Daphnia magna*; Gam-96: LC_{50}-96 h for *Gammarus* spp. Daph/G: ratio LC_{50} *Daphnia*/LC_{50} *Gammarus*, where *Daphnia* proved more sensitive, that is, ratio below 1. D/Gam: ratio LC_{50} *Daphnia*/LC_{50} *Gammarus*, where *Gammarus* proved more sensitive, that is, above 1. Daph-96(2): LC_{50} *Daphnia* 48 h/2 for correction of increase in toxicity with increasing exposure time. D/G corr: corrected ratio *Daphnia/Gammarus*.

Substance (μg/L)	Daph-48	Gam-96	Daph/G	D/Gam	Daph-96 (2)	D/G-corr
Metals						
Cadmiumchloride	25	19		1,3	12,5	0,6
Copper	9	37	0,2		4,5	0,12
Chromiumchloride	108000	64000		1,7	54000	0,8
Coppersulfate	180	20		9	90	4,5
Copperchloride	825000	12000		68	412500	34,4
Leadnitrate	43000	124		364	21500	173,4
Mercurychloride	93	9		10	46,5	5,2
Nicklechloride	6700	15000	0,4		3350	0,2
Zinknitrate	868000	2000000		4,3	434000	2,2
Zinkchloride	93800	10200		9	46900	4,5
Organochlorine insecticides						
Aldrin	33	17		2	16,5	0,9
Aramite	12000	60000	0,2		600	0,01
Dieldrin	79	640	0,1		39,2	0,06
Endrin	50	6		8	25	4,2
Methoxychlor	0,8	0,9	0,8		0,4	0,4
Tetradifon	140	110		1,2	70	0,6
Toxaphene	10	30	0,3		5	0,2
Organophosphate insecticides						
Azinphos methyl	1	0,1		10	0,5	5
Dichlorphos	770	0,5		1540	385	770
Diazinon	1	10	0,1		0,5	0,05
Chlorpyrifos	0,25	0,3	0,8		0,125	0,42
Dimethoate	2900	200		14,5	1450	7,25
Dioxathion	1	8	0,12		0,5	0,06
Disulfoton	100	240	0,4		50	0,2
Ethion	60	2		30	30	15
Fenthion	6	9	0,6		3	0,3
Imidan	6	2		3	3	1,5
Parathion	7	0,6		11,6	3,5	5,8
Phorate	31	5		6,2	16,5	3,1
Phosphamidon	15	16	0,9		7,5	0,45
Temephos	10	82	0,12		5	0,06
Trichlorophon	180	50		3,6	90	1,8
Carbamate insecticides						
Aminocarb	190	12		15,8	95	7,9
Baygon	29	40	0,7		15	0,35
Carbaryl	60	20		30	30	15
Further Pesticides						
Atrazine	6900	5700		1,2	3450	0,6
Bensulide	92000	3300		27,8	46000	13,9
Dicamba	110000	5800		18,9	55000	9,4
Dichlone	14	2		7	7	3,5
Diuron	1000	1000		1	500	0,5
2-4-D	184000	76000		2,4	9200	1,2
Endothall	223000	100000		2,23	111500	1,1
Eptam	14000	35000	0,4		7000	0,2

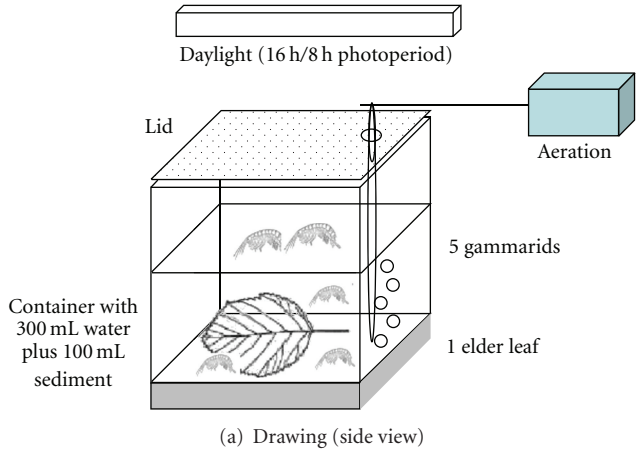
Table 1: Continued.

Substance (μg/l)	Daph-48	Gam-96	Daph/G	D/Gam	Daph-96 (2)	D/G-corr
Molinate	25000	7600		3,2	1250	1,6
Paraquat	6100	18000	0,3		3050	0,1
Picloram	68000	48000		1,4	3400	0,7
Propanil	5000	34000		1,5	2500	0,75
Silvex BEE	7000	740		9,4	3500	4,7
Simazine	100000	21000		4,7	50000	2,3
3.4 DCA	1000	17000	0,05		500	0,025
Pirimicarb	40	48	0,8		20	0,4
Allethrin	20	11		1,8	10	0,9
Pyrethrum	500	12		42	250	21
Terbutryn	2600	4000	0,6		1300	0,3
Fenoxycarb	600	4000	0,15		300	0,075
Lindan	1600	125		12,8	800	6,4
Deltamethrin	0,3	0,005		60	0,15	30
Tebuconazole	4200	1600		2,6	2100	1,3
Imidacloprid	85000	270		314,8	42500	157,4

altered abundance of the species in the ecosystem [11]. Behavioral responses seem to be of similar sensitivity and efficiency as biochemical and physiological responses [10] and can be recorded both automatically and quantitatively, thus allowing the field of behavioral ecotoxicology to expand [12, 13]. Behavioral alterations have been linked to changes in acetylcholinesterase activity under exposure to neurotoxic pesticides in several aquatic species including gammarids [10, 14, 15].

Feeding rate is a sensitive sublethal endpoint compared to community-related measures which require changes in species composition before an impact is detected [16, 17]. Bloor and Banks [18] compared *in situ* and *ex situ* feeding assays with both the pollution-sensitive *G. pulex,* and the pollution tolerant *A. aquaticus*. Both, mortalities and feeding rates followed similar trends during the *in situ* and *ex situ* tests, but the response of test animals was amplified during *in situ* testing. Maltby et al. [17] found a strong positive correlation between *in situ* feeding rate of gammarids and macroinvertebrate diversity and a biotic index. Thus, the new GamTox might fill an important gap in current water quality assessment as it represents a measure for xenobiotic stress ("ecotoxicological" water quality assessment), which has neither been covered by existing biological water quality assessment methods (biodiversity, biotic indices) nor by chemical assessment methods.

The aim of this study was to develop a low-cost test protocol for a multimetric sublethal toxicity test with *Gammarus* spp. based on feeding activity, behavior and survival as well as biochemical biomarkers for both *in* and *ex situ* application to be included in routine water quality monitoring programmes.



(a) Drawing (side view)

(b) Photograph (view from the top, photo A. Gerhardt)

Figure 1: Experimental unit for the *ex situ* test: (a) drawing, (b) photograph.

2. Methods

2.1. Origin of the Test Organisms and Culture. Gammarus spp. can be collected in a reference stream, being rather unpolluted, in all size classes throughout the year. Chemical water quality parameters and biological bioassessment data ("good water quality" according to EU-WRRL) might be available from routine biomonitoring programmes by the

Figure 2: Experimental setup *in situ* (Photo: A. Gerhardt). View from top: four test chambers (15 × 5 cm) are placed on the sediment of the stream in flow direction, attached with ropes to steel poles in the sediment and on the banks. Each chamber contains 10 gammarids and 2 elder leaves. The test chambers can be connected to the MFB.

water authorities for the choice of appropriate reference conditions. Field-taken organisms need to be acclimated to laboratory test conditions for at least one week prior to testing.

Gammarus spp, taken from the field, might also be maintained during several weeks or even cultured in aerated aquaria with unfiltered stream water for several months. Successful cultures have been described in several previous papers (e.g., [19–21]).

In order to simplify the different protocols for culturing, the following procedure has been used successfully. Ca. 100 *Gammarus* spp. of mixed gender and size classes are collected from the field, and placed in a 20 L glass aquarium with a layer of 2 cm sediment (sand and pebbles) including organic matter (leaf packs) from the site of origin. An aquarium pump with filter (Eheim aquaball 2400, 45 L) is placed in the aquarium within a net cage (0.1 mm mesh size) for water circulation and removal of high loads of particles. Every week the filter is cleaned and 50% of the water in the aquarium is exchanged by adding new stream water after acclimation to room temperature (between 16 and 22°C). Once a week, 10 leaves of elder are added, twice a week the animals are fed additionally with frozen chironomids *at libitum*. The culture is kept for several months at room temperature in a dark room with daylight neon illumination with a photoperiod of 16-hour light/8 h night. Once a month the animals are checked visually, precopula pairs and small juveniles have been observed frequently, a sign of a healthy culture. Each time animals are taken out for experiments, new animals from the reference stream are added to refresh the gene pool of the culture.

2.2. Experimental Design Ex Situ. The experiments are executed under the same conditions as chosen for the culture concerning light and temperature (15–22°C). The exposures are arranged in rectangular white-opaque hard-polyethylen containers (PE, 400 mL: 10 cm × 10 cm × 6 cm in high); the exposure lasts for 12 days without any renewal of water, food, or faeces (Figure 1). In each container 5 animals are placed and 3 replicates per treatment are set up, this test design represents the minimum set up concerning statistical evaluation. The PE containers contain either only water for tests with surface and waste waters or water and sediment (300 mL water, 100 mL sediment) for tests with toxic sediments from, for example, waste disposals and overlaying water. The water in each container is continuously aerated through a pipette to reach 100% oxygen saturation. One preconditioned elder leaf is added to each container as both food source and substrate. Every 4 days survival, behavior (either: response to prodding with a forceps, or: recording in the Multispecies Freshwater Biomonitor) and feeding rate (0, 25, 50, 75, 100% of leaf consumption) is monitored. Leaf consumption is visually monitored either by eye or by photographs in an easy manner as the animals produce feeding traces, they sceletize the leaf. The containers are covered with a PE lid to avoid evaporation. Control survival and feeding rates of the animals of >80% after 12 days of exposure in the reference water are regarded as quality criteria for the experimental design.

2.3. Experimental Design In Situ. In situ exposures are arranged in test chambers of the Multispecies Freshwater Biomonitor (MFB) [12, 22] (Figure 2). Eight acrylic glass cylinders of 15 cm length and 5 cm inner diameter capped with screw rings on both ends, which contain a nylon net of 0.5 mm mesh size are exposed in the current flow and attached both in the sediment and on the banks using stainless steel poles. Each test chamber contains 10 animals and 2 preconditioned elder leaves. Every 4 days survival and behaviour of the animals are recorded with the Multispecies Freshwater Biomonitor for 30 minutes (generating 3 subsequent online measurements) operated by a car battery [23, 24]. In these conditions the animals can be monitored in their exposure cages in the stream without disturbance at several subsequent occasions until the end of the experiments after 12 days. At the end of the experiment the animals are manually collected and counted, the feeding rate is noted. With this setup, experiments can be performed at low and normal water levels in small wadeable streams with permanent flow. In case of rapidly increasing water levels due to heavy rainfall events and floods, the equipment has to be checked more frequently in the field.

2.4. Multispecies Freshwater Biomonitor (MFB). The described experiments can be executed in the test chambers, with or without the MFB, however, without the MFB; the animals need to be checked manually every 4 days in the cages, and this might create additional stress for the animals. The MFB has already been used in several *in situ* applications. Several *ex situ* and *in situ* tests have been conducted with the fully automated real-time-based Multispecies Freshwater Biomonitor, developed by Gerhardt et al. [22]. The system is based on quadrapole impedance conversion technique that simultaneously records several behavioral parameters of a wide range of aquatic organisms, for example, different

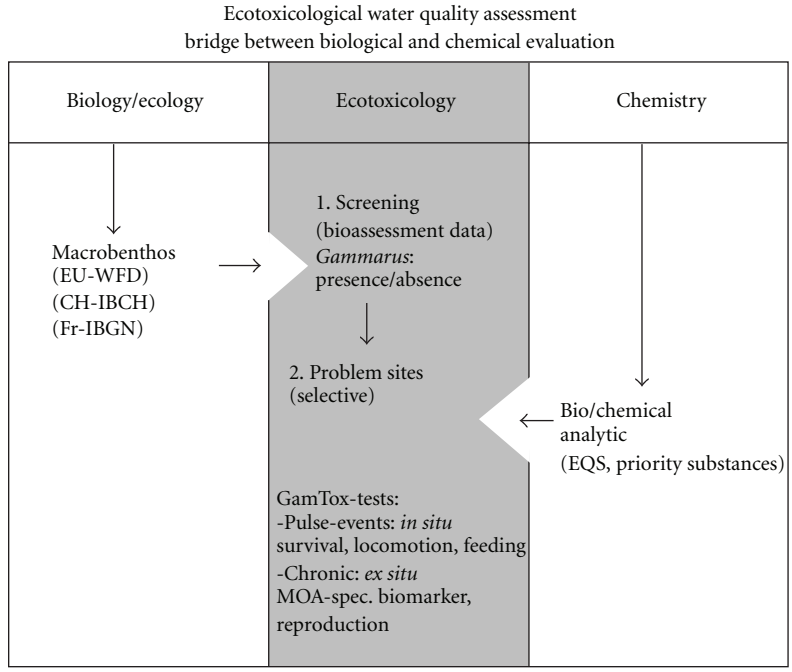


Figure 3: Integrated concept for water quality assessment with a new ecotoxicological module.

crustaceans, insect larvae, oligochaets, molluscs, tadpoles, and several fish species. During exposure, the organisms move freely between two pairs of electrodes on each sidewall of a test chamber, which receives unfiltered stream water or exposure water [22]. The organism's behavior is expressed as movements that lead to changes in an electrical field and these are measured as changes in the impedance of the system. For example, (1) locomotion: swimming and crawling results in irregular amplitudes and frequencies, (2) resting: small signals that cannot be separated from background noise, (3) ventilation: regular, high-frequency movements with, for example, pleopods to establish a constant water flow across the gills, and (4) feeding: species-specific patterns for grazing, filtering, and hunting. The impedance converter proved to be a sensitive and quantitative tool for use in behavioral, ecological and ecotoxicological studies, which makes it a promising tool for continuous biomonitoring purposes [25], as proven with *Gammarus* spp. exposed to a copper pulse [12] or in river monitoring stations along the rivers Meuse (NL), Aller (GER) and Rhine (F) [10].

3. Results

The new GamTox test protocol was validated in laboratory exposures with different types of polluted surface water [26]: (1) leakage from the waste disposal containing solvents, caused increased mortality and decreased feeding activity of the gammarids. The stream water showed elevated levels of iron and Ammonium and the biological water quality class was described as unsatisfactory (according to the European WFD class 4). (2) the effluent from a municipal waste water treatment plant containing pesticides (WWTPs) caused high mortality and decreased feeding activity of *Gammarus* fossarum as well as >20% AChE inhibition, a toxicity threshold previously defined [14, 15, 27].

GamTox *in situ* validation was conducted in a small reference stream with locally abundant *Gammarus* spp. Five tubes with each 10 organisms and two elder leaves were exposed *in situ* for 12 days. After 12 days survival and feeding rates of the gammarids resident in the stream were >85% in all chambers.

4. Discussion

The GamTox test protocol proved to be easy and sensitive towards chloroalkanes, aromatic compounds, and pesticides in the laboratory assays regarding survival and sublethal test parameters such as feeding behavior and AChE inhibition. A first test of the protocol *in situ* showed the practicability of the test in a reference stream, further test validation in streams polluted by pesticide pulses are currently being performed and will be published separately.

In previous tests with *Gammarus fossarum* studying survival and behavior, however, with different exposure times, *Gammarus fossarum* proved more sensitive towards $AgCl_2$-exposure (0.7 mg/L) than other test species (*Pseudokirchneriella subcapitata, Vibrio fisheri*) and especially locomotory activity recorded in the Multispecies Freshwater Biomonitor revealed effects already after a few hours of exposure at concentration levels which proved to be lethal after 7 days of exposure [28]. Sediment pore water from a solid pulp waste disposal was tested with different standard toxicity tests and compared with *Gammarus fossarum* acute toxicity test (24-hour survival, feeding activity). The waste disposal was polluted with PCBs, PAHs, and Cd. While no effects were seen in algae (*P. subcapitata*) growth and photosynthesis,

Gammarus fossarum showed effects already after 24 hours of exposure with increasing locomotion, a sign of avoidance behavior. Chronic population-relevant effects of this disposal could also been seen after 8 days (*Ceriodaphnia dubia*: reproduction), after 10 days (*Chironomus riparius*: survival) and after completed emergence of *Chironomus riparius* [29]. This indicates, that *Gammarus*' behavior can be used as early warning test for ecologically relevant evaluation of both acute and chronic toxicity of even other freshwater invertebrate species. This validation was the base to continue with simplifying and automation of toxicity testing with *Gammarus*, as it is described here in the new test protocol for both *in* and *ex situ* testing. This new test protocol needs further validation by the water authorities performing water quality bioassessment in their routine monitoring programmes.

The following new concept for an ecotoxicological water quality assessment as part of a traid-based integrated water quality monitoring is now proposed (Figure 3). In a literature study [27] it could be shown that *Gammarus* spp. react in a very sensitive way towards pesticide pulses, esp. neurotoxic insecticides, and locomotory behavior (drift, locomotion) seems to be the most appropriate test parameter. In some small streams polluted temporarily by pesticide pulses, gammarids were reported missing or their population densities were decreasing which was observed during several years of routine biomonitoring. As gammarids are reported indicators for the saproby class 2 (beta-mesosaprob), corresponding to "good" water quality according to the EU-WFD, the decline or presence/absence of gammarids could be a fist indicator of pesticide pollution. This evaluation can be done by just re-evaluating existing bioassessment data over the past times. Additionally, the SPEAR index (species at risk) can be calculated from macrozoobenthos data (http://www.systemecology.eu/SPEAR/index.php). Like this, problem sites might be discerned where GamTox as described above can easily be conducted *in* or *ex situ*, without the need of a sophisticated laboratory, as both the toxicity test and the culture can be managed at room temperature and no expensive equipment is required for the test. GamTox aims to verify the observed stress (e.g., *Gammarus* presence/absence, macrobenthos, SPEAR, and chemistry) as being based on ecotoxicological effects. Additionally, manpower can be reduced by executing GamTox in a fully automated manner in the MFB. In case GamTox (survival, feeding, and locomotion) indicates toxicity further tests such as specific biomarkers and/or reproduction might be carried out if bio/chemical water analyses reveal elevated concentration levels of pesticides and/or surpass the proposed environmental quality standards.

Acknowledgments

Olivier Geffard (Cemagref, Lyon, France) is gratefully acknowledged for performing the AChE analysis on gammarids. AquaPlus (Zug, Switzerland) is thanked for financial support of parts of the experimental work.

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