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The usefulness of *Gasterosteus aculeatus* - the three-spined stickleback - as a testorganism in routine toxicity tests

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SUMMARY

Most of the small fish species recommended to be used in short term and early life stage tests by the OECD are only manageable in laboratory studies and not in field experiments in countries with a temperate climate.
In order to find a fish species which has not this restriction, research was carried out with the three-spined stickleback (Gasterosteus aculeatus). The three-spined stickleback is very common in Europe, parts of Asia and Northern America between the 35th and 70th degree of latitude and occurs in a wide variety of watertypes.

The study of the usefulness of the three-spined stickleback as a testorganism in routine toxicity tests was based on extended earlier studies in the Netherlands. In this research four tropical fish species (Brachydanio rerio, Jordanella floridae, Oryzias latipes and Poecilia reticulata) were used in short term and long term toxicity tests with several chemical agents (potassiumdichromate, diisopropylamine, di(2-ethylhexyl)phtalate, 2,4-dinitrotoluene, 2,6-dimethylquinoline, 2,4-dichloroaniline, tricresylphosphate and tetrapropylenebenzenesulphonate). The sensitivity of the stickleback for the above mentioned chemicals was compared with these four fish species.

For the comparison of the results obtained from short term toxicity tests the 96-h LC50- and 96-h NOEC-values were used (E = mortality, abnormal behaviour and appearance). For the comparison of the results obtained from long term toxicity tests (including early life stage tests) the lowest NOEC-values were used (E = mortality, sublethal effects during embryonic development or growth). The results show that G. aculeatus is a sensitive fish species in short term and early life stage toxicity tests.

Because of above mentioned characteristics and the fact that the stickleback is rather simple to rear, it is recommended to place G. aculeatus on the list of recommended species in OECD-test guidelines or EC-test methods.

SAMENVATTING

De meeste, door de OECD aanbevolen, kleine vissoorten voor kortdurend toxiciteitsonderzoek en/of early life stage testen zijn in landen met een gematigd klimaat alleen te gebruiken in laboratoriumstudies en niet in veldstudies.
Om een geschikte vissoort te vinden die deze beperking niet heeft, is er onderzoek uitgevoerd met de driedoornige stekelbaars (Gasterosteus aculeatus). Deze vis komt algemeen voor in Europa, grote delen van Azie en Noord Amerika tussen de 35e en 70e breedtegraad in veel verschillende soorten water.

Het onderzoek naar de bruikbaarheid van de driedoornige stekelbaars als toetsorganisme in routine toxiciteitsonderzoek is gebaseerd op eerder uitgevoerd onderzoek in Nederland. In dit onderzoek werden vier tropische vissoorten (Brachydanio rerio, Jordanella floridae, Oryzias latipes en Poecilia reticulata) gebruikt in zowel kort- als langdurend toxiciteitsonderzoek met verschillende chemische stoffen (kaliumdichromaat, diisopropylamine, di(2-ethylhexylftalaat, 2,4-dinitrotolueen, 2,6-dimethylquinoline, 2,4-dichlooraniline tricresylfosfaat en tetrapropyleenbenzeensulfonaat). De gevoeligheid van de stekelbaars voor deze verbindingen is vergeleken met de vier bovengenoemde vissoorten.

Voor de vergelijking van de resultaten uit het kortdurend toxiciteitsonderzoek zijn de LC50- en NOEC-waarden (96 uur) gebruikt (E = mortaliteit, abnormaal gedrag en afwijkend uiterlijk). Voor de vergelijking van de resultaten uit het langdurend toxiciteitonderzoek (inclusief early life stage testen) werden de laagste NOEC-waarden gebruikt (E = mortaliteit, sublethale effecten tijdens het embryonale stadium of groei). Uit de resultaten blijkt dat G. aculeatus een gevoelige vissoort is in zowel kortdurend toxiciteitsonderzoek als early life stage testen.

Op grond van bovengenoemde kenmerken en het feit dat de stekelbaars eenvoudig te kweken is, is het aan te bevelen G. aculeatus te plaatsen op lijst van aanbevolen toetsorganismen in OECD-test guidelines en EG-test methoden.

1 INTRODUCTION

By order of the Ministry of Housing, Physical Planning and Environment, research was carried out to select a group of tests to assess the effects of chemical agents on the aquatic environment (Adema et al., 1981; Adema et al., 1983; Canton et al., 1984). This research was focussed on internationally acccepted groups of organisms: algae, crustaceans and fish. According to these authors long term toxicity tests with several different species should be part of the tests. They suggested, apart from other tests, to use an egg-laying fish to observe effects on the embryonic development and survival and growth of the juvenile fish (so called "early life stage" test).

In the above mentioned study only tropical fish species were examined: Oryzias latipes (medaka), Jordanella floridae (flagfish), Brachydanio rerio (zebrafish), all egg-laying fish and Poecilia reticulata (guppy), a viviparous fish species. Most of these fish were, among others, recommended to be used in short term (OECD, 1984) and early life stage toxicity (OECD, 1987) tests by the OECD. These species are manageable in laboratory studies, however, they can not be used in field experiments in countries with a temperate climate. To meet this problem the usefulness of the three-spined stickleback (Gasterosteus aculeatus) as a testorganism in routine toxicity tests was studied. This species can be found between the 35th and 70th degree of latitude on the northern hemisphere in Europe, parts of Asia and Northern America, in several watertypes such as ditches, rivers, lakes and even the marine environment. The primary objective of this study was to compare the sensitivity of G. aculeatus towards eight chemical agents with the above mentioned fish species in short term toxicity tests and early life stage tests. Additionally a limited literature-search was carried out to compare the sensitivity of G. aculeatus with several other fish species.

2 MATERIAL EN METHODS

2.1 Test organisms

The rearing of G. aculeatus in the laboratory was started with adults obtained from the Zoological Laboratory, University of Leiden (Department of Ethology) (for details see appendix a). The eggs and young fish (4 to 5 weeks old) used in the experiments were at least of the 2nd generation of fish maintained under standardized conditions.

2.2 Toxicity tests

Short term toxicity tests were carried out with young fish (4 to 5 weeks) to determine LC50, EC50, NOLC and NOEC after 24-, 48-, 72- and 96-h. Tests with respect to early life stages were carried out on eggs (less than 6 hours old) to determine NOEC (embryonic stage) and LC50, EC50, NOLC, NOEC (mortality, sublethal effects and growth) after 4 weeks (fish stage).
The tests were conducted according to the methods outlined by Adema et al. (1981). The criteria to assess and scale the sensitivity and the experimental conditions are listed in Table 1.

Dissolved oxygen and pH were measured in the control and in the highest test concentration after 0- and 48-h (short term tests) and weekly after 0-, 48- and 72-h (early life stage tests). Dissolved oxygen was measured with a Beckman 0260 oxygen analyzer; pH was measured with a Philips 9409 pH analyzer.

2.3 Toxicants and analyses

The tests were carried out with the eight chemicals used in Adema et al. (1981): potassiumdichromate (Merck, $\geq$ 99.9 %), diisopropylamine (Fluka, puriss.), di(2-ethylhexyl)phtalate (Merck, 97 %), 2,4-dinitrotoluene (Merck, 98 %), 2,6-dimethylquinoline (Janssen Chemica, 98 %), 2,4-dichloroaniline (Fluka, $\geq$ 99 %), tricresylphosphate (Fluka, pract.) and tetrapropylenebenzenesulphonate (Merck, 5 %). The initial concentrations in the test chambers of the lowest and the highest test concentrations were confirmed by chemical analyses. The methods for these analyses are described in Adema et al. (1981). They have shown that the test concentrations are at least 70 % of nominal values 72 h after preparation of the solutions.

2.4 Calculations

LC50- and EC50-results were calculated according to Litchfield and Wilcoxon (1949). Differences between the growth at different concentrations were tested by means of a Student-T test which resulted in the NOEC-growth (length and wet weight). NOLC and other NOEC values were directly derived from the experiments.

3 RESULTS AND DISCUSSION

3.1 Results

Results of the short term toxicity tests and the early life stage tests for G. aculeatus are listed in Table 2 and Table 3 respectively. These results are given as nominal concentrations and actual concentrations. The results of the chemical analyses are given in Table 4 (short term tests) and Table 5 (early life stage tests). The analyses of di(2-ethylhexyl)phtalate and tricresylphosphate in the early life stage tests have failed; the results of these tests are therefore reported as nominal concentrations.

3.2 Comparison of the sensitivity of G. aculeatus in short term toxicity tests

The LC50 and NOEC values obtained in this study with G. aculeatus in short term toxicity tests were compared with those of B. rerio, J. floridae, O. latipes and P. reticulata reported in Adema et al. (1981). The 96-h LC50 values of those five fish species are listed in Table 6, 96-h NOEC values are listed in Table 7. Table 6 shows that the stickleback is as sensitive as or even more sensitive in the short term toxicity tests in comparison with the other fish species mentioned. The same conclusion can be drawn from Table 7, except for diisopropylamine. The stickleback is slighty less sensitive (a factorial difference of max. 3.2) for this chemical agent.

A comparison with some other fish species can be found in Jop et al. (1986). They tested the toxicity of hexavalent chromium with four fish species. For three of these species the 96-h LC50's were approximately the same: Cyprinodon variegatus 23.2 mg/l, Pimephales promelas 39.8 mg/l and G. aculeatus 42.6 mg/l. Lepomis macrochirus was the least sensitive species tested, 191.9 mg/l. The age of the fish used in their experiments was 2 to 3 months; in the study reported here the 96-h LC50 is 30.1 mg/l (calculated from the value for potassiumdichromate, Table 2). The slight difference between the two values may be caused by the difference in age of the fish, but falls within the reproducibility of this type of experiments. Another comparison with some other species can be made using the results of Johnson and Finley (1980). They reported the 96-h LC50 for bluegills towards tricresylphosphate to be 150 ug/l, for rainbow trout 260 ug/l, for yellow perch 502 ug/l and for channel catfish 803 ug/l. The 96-h LC50 for the stickleback in the study reported here was found to be 510 ug/l (Table 2). Liu et al. (1983) reported a 96-h LC50 for 2,4-dinitrotoluene with fathead minnow of 32.8 mg/l. In the study reported here G. aculeatus is the most sensitive species in the short term test with 2,4-dinitrotoluene. The 96-h LC50 was found to be 6.4 mg/l. (Table 6). Calamari et al. (1980) found the 96-h LC50 for rainbow trout towards diisopropylamine to be 196 mg/l. For the stickleback the 96-h LC50 proved to be 840 mg/l (this report).

A comparative study with stickleback, rainbow trout and fathead minnow has been conducted by Smith et al. (1985). They concluded that rainbow trout and fathead minnow appear more sensitive to fluoride ion then do sticklebacks although the overall range of 96-h LC50 values observed (180 to 460 mg/l, depending on species and conditions) varied by a factor of only 2.6. Katz (1961) carried out short term toxicity tests on thirteen insecticides with three species of salmonids, among which rainbow trout, and sticklebacks (0.5 % and 2.5 % salinity). The results show, indicatively, that there is no striking difference in 96-h median tolerance limits obtained with marine sticklebacks from the data obtained with salmonids. Another, indicative, comparison with some other species is given by Lemma and Yau (1974). They showed in a short term experiment (24-hour, LC90) with G. aculeatus, Tilapia mossambica and Carassius auratus that the stickleback was the most sensitive of these species for five molluscicides tested.

3.3 Comparison of the sensitivity of G. aculeatus in long term toxicity tests

The NOEC-values obtained from long term toxicity tests including early life stage tests are listed in Table 8. The NOEC-values for embryonic development are listed in Table 9. Only nominal concentrations are reported. Table 8 shows that the stickleback is 5.6 times less sensitive for potassiumdichromate and diisopropylamine than the most sensitive species reported here. For the other chemicals it is as sensitive as or more sensitive than the other species tested except for tricresylphosphate. For this chemical the stickleback is at least 18 times more sensitive than the other species.

The sensitivity of the embryonic stage of G. aculeatus has been compared with that of the other egg-laying fish, B. rerio, J. floridae and O. latipes. Table 9 shows that, for this toxicological endpoint, G. aculeatus is most of the times as sensitive as or more sensitive (for tricresylphosphate 10 times more) than the other species.

A comparison with some other species can be made using the data of Bailey et al. (1984). They performed early life stage tests with 2,4-dinitrotoluene using rainbow trout, channel catfish and fathead minnow as testorganisms. They found a NOEC-growth (60 days) for rainbow trout of 0.23 mg/l, for fathead minnow a NOEC-growth (30 days) of 3.1 mg/l and for channel catfish a NOLC (30 days) of < 3.4 mg/l. These studies were carried out in very soft water (ca. 32 mg/l $CaCO_3$). In Table 3 the NOLC (35 days) for 2,4-dinitrotoluene with sticklebacks is 1.4 mg/l and the NOEC-growth (35 days) is 0.77 mg/l. Mehrle and Mayer (1976) have studied the toxicity of di(2-ethylhexyl)phtalate in an early life stage test for rainbow trout. The NOLC at 24 days after hatching was 5 ug/l. In the study reported here the NOLC for sticklebacks is $\geq$ 320 ug/l (Table 3). Macek and Sleight (1977) refer to the results of Brungs (1969). He did find a NOEC-growth (30 days) in soft water for S. gairdneri of 0.384 mg chromium/l. This report gives a NOEC-growth (35 days) in hard water for G. aculeatus of 6.4 mg chromium/l (calculated from the value for potassiumdichromate, Table 3).

These data show that G. aculeatus is a sensitive species compared with some other, routinely used, fish species. Compared with S. gairdneri, a non-tropical species, the stickleback appears to be less sensitive. In the case of using non-tropical species the advantages of the stickleback are its availability of all life stages during the whole year (which is not for the eggs and juveniles of rainbow trout), its easy managebility and its natural occurrence in several watertypes which is important for field studies.

4 CONCLUSIONS AND RECOMMENDATIONS

From this study it can be concluded that G. aculeatus is a sensitive fish species in short term tests and early life stage tests. Its sensitivity is among that of O. latipes, J. floridae, B. rerio and P. reticulata. Compared with literature data on S. gairdneri, G. aculeatus appears to be less sensitive.

The stickleback is simple to rear. It is a manageable species, even in the egg-stage. These characteristics make G. aculeatus suitable for toxicity testing in laboratories. Its temperature demands and the fact that this species occurs in a wide variety of watertypes makes the stickleback also very suitable for use in outdoor ecosystems like experimental ponds or bioassays under temperate climate conditions.

Because of these characteristics it is recommended to place G. aculeatus on the list of recommended species in OECD-test guidelines or EC-test methods.

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LITERATURE

Adema, D.M.M., J.H. Canton, W. Slooff en A.O. Hanstveit, (1981).
Onderzoek naar een geschikte combinatie toetsmethoden ter bepaling van de aquatische toxiciteit van milieugevaarlijke stoffen.
Rapport nr : RIV 627905 001, National Institute of Public Health and Environmental Protection, Bilthoven.

Adema, D.M.M., J. Kuiper, A.O. Hanstveit and J.H. Canton, (1983).
Consecutive system of tests for assessment of the effects of chemical agents in the aquatic environment.
In : Pesticide chemistry : Human welfare and the environment, J. Miyamoto et al., eds., Pergamon Press, Oxford, pp 537-544.

Bailey, H.C., R.J. Spanggord, H.S. Javitz and D.H.W. Liu, (1984).
Toxicity of TNT wastewaters to aquatic organisms: Volume 4. Chronic toxicity of 2,4-dinitrotoluene and condensate water.
SRI International; report number LSU-4262, Menlo park California.

Brungs, W.A. (1969).
Transactions.
American Fisheries Society, 98, (2), pp 272-279.

Calamari, D., R. Da Gasso, S. Galassi, A. Provini and M. Vighi, (1980).
Biodegradation and toxicity of selected amines on aquatic organisms.
Chemosphere, 9, pp 753-762.

Canton, J.H., D.M.M. Adema en D. de Zwart, (1984).
Onderzoek naar de bruikbaarheid van een drietal eierleggende vissoorten in routine toxiciteitsonderzoek.
Rapportnummer : RIVM 668114 002, National Institute of Public Health and Environmental Protection, Bilthoven.

Johnson, W.W. and M.T. Finley, (1980).
Handbook of acute toxicity of chemicals to fish and aquatic invertebrates.
Fish and Wildlife Service.

Jop, K.M., J.H. Rodgers Jr., P.B. Dorn and K.L. Dickson, (1986).
Use of hexavalent chromium as a reference toxicant in aquatic toxicity tests.
In : Aquatic toxicology and environmental fate : ninth volume, ASTM STP 921, T.M. Poston and R. Purdey, eds., American Society for Testing and Materials, Philadelphia, pp. 390-403.

Katz, M. (1961).
Acute toxicity of some organic insecticides to three species of salmonids and to the threespine stickleback.
Trans. Am. Fish. Soc., 90, (3), pp 264-268.

Lemma, A. and P. Yau, (1974).
Studies on the molluscicidal properties of Endod (Phytolacca dodecandra): II. Comparative toxicity of various molluscicides to fish and snails.
Ethiop. Med. J., 12, pp 109-114.

Litchfield, J.T. Jr. and F. Wilcoxon, (1949).
A simplified method of evaluating dose-effect experiments.
J. Pharm. Exp. Therap. 96 : pp 99-113.

Liu, D.H.W., H.C. Bailey and J.G. Pearson, (1983).
Toxicity of a complex munitions wastewater to aquatic organisms.
In : Aquatic toxicology and hazard assessment: sixth symposium, ASTM STP 802, W.E. Bishop, R.D. Cardwell and B.B. Heidolph, eds., American Society for Testing and Materials, Philadelphia, pp 135-150.

Macek, K.J. and B.H. Sleight, (1977).
Utility of toxicity tests with embryos and fry of fish in evaluating hazards associated with the chronic toxicity of chemicals to fishes.
In : Aquatic toxicology and hazard evaluation, ASTM STP 634, F.L. Mayer and J.L. Hamelink, eds, American Society for Testing and Materials, pp 137-146.

Mehrle, P.M. and F.L. Mayer, (1976).
Di-2-ethylhexyl phtalate: residue dynamics and biological effects in rainbow trout and fathead minnows.
In : Proceedings of University of Missouri's Annual conference on trace substances in environmental health; 10, (recd 1977), pp 519-524.

OECD, (1984).
Fish, acute toxicity test (203).
In : Guidelines for testing of chemicals, OECD, Paris.

OECD, (1987).
Fish, early life stage toxicity test.
In : Guidelines for testing of chemicals, Eco 87.2, (draft), OECD, Paris.

Smith, L.R., T.M. Holson, N.C. Ibay, R.M. Block and A.B. De Leon, (1985).
Studies on the acute toxicity of fluoride ion to stickleback, fathead minnow and rainbow trout.
Chemosphere, 14, (9), pp 1383-1389.

Table 1. Experimental conditions and toxicological criteria in short term toxicity tests and early life stage tests with G. aculeatus.

CONDITIONS AND CRITERIA	SHORT TERM TOXICITY TESTS	EARLY LIFE STAGE TESTS
Age	4 to 5 weeks	< 6 hours (eggs)
Exposure time	96 hours	ca. 35 days
Number of organisms per group	10 (in duplicate)	25 (singular)
Testvolume	1 litre(*) in allglass vessels	eggs : 1 litre(*) fish : 2 litre(*) in allglass vessels not aerated
Lighting	circadic 16 hours light and 8 hours dark	circadic 16 hours light and 8 hours dark
Temperature	19 ± 1 °C	19 ± 1 °C
Dosing	semistatic	semistatic
Renewing rate	once at 48 hours	3 x a week on mon., weds, and friday
Food	none	first three days after hatching B. rubens (ad libitum); day 4 - 21: 3x a day artemia's; day 21 - 28: 2x a day artemia's and 1x a day dry food
Criteria	mortality, swimming behaviour and appearance	mortality and embryonic development (eggs) mortality, swimming behaviour, appearance and growth of the fry

*) Test medium : DSW, hardness 11,7 °DH ; pH 8.2 ± 0.2 (see also appendix b)

Table 2. Summary of the results (in mg/l) of the short term toxicity tests with G. aculeatus.

Compound	LC50/EC50 NOLC/NOEC (*)	24 hr nominal	24 hr actual	48 hr nominal	48 hr actual	72 hr nominal	72 hr actual	96 hr nominal	96 hr actual
potassiumdichromate	LC50	110	107	90	87	85	82	85	82
	EC50	75	73	42	41	42	41	42	41
	NOLC	56	54	56	54	56	54	32	31
	NOEC	56	54	32	31	32	31	32	31
diisopropylamine	LC50	870	827	840	798	840	798	840	798
	EC50	420	399	420	399	420	399	420	399
	NOLC	320	304	320	304	320	304	320	304
	NOEC	320	304	320	304	320	304	320	304
di(2-ethylhexyl)-phtalate	LC50	> 0.32	> 0.30	> 0.32	> 0.30	> 0.32	> 0.30	> 0.32	> 0.30
	EC50	> 0.32	> 0.30	> 0.32	> 0.30	> 0.32	> 0.30	> 0.32	> 0.30
	NOLC	≥ 0.32	≥ 0.30	≥ 0.32	≥ 0.30	≥ 0.32	≥ 0.30	≥ 0.32	≥ 0.30
	NOEC	≥ 0.32	≥ 0.30	≥ 0.32	≥ 0.30	≥ 0.32	≥ 0.30	≥ 0.32	≥ 0.30
2,4-dinitrotoluene	LC50	> 16	> 16	7.0	6.9	6.6	6.5	6.4	6.3
	EC50	2.4	2.4	1.3	1.3	1.3	1.3	1.3	1.3
	NOLC	≥ 16	≥ 16	1.8	1.8	1.8	1.8	1.8	1.8
	NOEC	1.8	1.8	1.0	0.98	1.0	0.98	1.0	0.98
2,6-dimethyl-quinoline	LC50	13	13	13	13	13	13	13	13
	EC50	1.3	1.3	0.75	0.73	0.75	0.73	0.75	0.73
	NOLC	10	9.7	10	9.7	10	9.7	10	9.7
	NOEC	1.0	0.97	0.56	0.54	0.56	0.54	0.56	0.54
2,4-dichloro-aniline	LC50	19	18	22	20	16	15	10	9.3
	EC50	4.2	3.9	2.4	2.2	2.4	2.2	2.4	2.2
	NOLC	5.6	5.2	5.6	5.2	5.6	5.2	5.6	5.2
	NOEC	3.2	3.0	1.8	1.7	1.8	1.7	1.8	1.7
tricresylphosphate	LC50	> 1.0	> 0.87	0.95	0.83	0.67	0.58	0.51	0.44
	EC50	0.66	0.57	0.42	0.37	0.24	0.21	0.20	0.17
	NOLC	0.32	0.28	0.32	0.28	0.18	0.16	0.18	0.16
	NOEC	0.32	0.28	0.32	0.28	0.18	0.16	0.18	0.16
tetrapropylene-benzenesulphonate	LC50	18	16	14	12	13	11	13	11
	EC50	13	11	13	11	7.5	6.5	7.5	6.5
	NOLC	10	8.7	10	8.7	10	8.7	5.6	4.9
	NOEC	10	8.7	10	8.7	5.6	4.9	5.6	4.9

(*) NOLC = No Observed Lethal Concentration

NOEC = No Observed Effect Concentration; effect = mortality, abnormal behaviour and appearance

Table 3. Summary of the results (in mg/l) of the early life stage tests with G. aculeatus.

Compound	NOEC embryonic stage		Fish stage: NOLC		Fish stage: NOEC mortality + sublethal effects (growth excluded)		Fish stage: NOEC growth		Fish stage: LC50		Fish stage: EC50 mortality + sublethal effects	
	nominal	actual	nominal	actual	nominal	actual	nominal	actual	nominal	actual	nominal	actual
potassium-dichromate	≥ 100	≥ 106	32	34	18	19	18	19	52	55	42	45
diisopropyl-amine	560	582	180	187	180	187	180	187	360	374	240	250
di(2-ethyl-hexyl)phta-late	≥ 0.32	----	≥ 0.32	----	≥ 0.32	----	≥ 0.32	----	> 0.32	----	> 0.32	----
2,4-dinitro-toluene	3.2	2.5	1.8	1.4	1.8	1.4	1.0	0.77	2.9	2.2	2.8	2.2
2,6-dimethyl-quinoline	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.9	1.9	1.9	1.9
2,4-dichloro-aniline	1.8	1.9	0.56	0.58	0.56	0.58	0.32	0.33	0.90	0.93	0.70	0.72
tricresyl-phosphate	0.0032	----	0.0010	----	0.0010	----	0.00032	----	0.0017	----	0.0013	----
tetrapropy-lenebenzene-sulphonate	10	9.4	5.6	5.3	5.6	5.3	1.0	0.94	11	10	6.8	6.4

Table 4. Nominal and actual concentrations (in mg/l) of the tested chemicals in the short term toxicity tests with G. aculeatus on t = 0 hr.

Compound	nominal concentration	actual concentration
potassiumdichromate	32 100	27 - 27 110 - 110
diisopropylamine	100 1000	90 - 100 890 - 1000
di(2-ethylhexyl)-phtalate	0.32 1.0	0.33 - 0.33 0.87 - 0.88
2,4-dinitrotoluene	1.0 16.4	0.84 - 1.21 15.0 - 15.9
2,6-dimethylquinoline	0.56 18	0.57 - 0.64 13.0 - 18.0
2,4-dichloroaniline	1.8 5.6	1.6 - 1.6 5.4 - 5.5
tricresylphosphate	0.10 1.0	87 % *
tetrapropylenebenzene-sulphonate	3.2 10	2.6 - 2.7 8.5 - 9.9

* mean of all values expressed as % peak area

Table 5. Nominal and actual concentrations (in mg/l) of the tested chemicals in early life stage tests with <u>G. aculeatus</u> on t = 0 hr.

Compound	nominal concentration	actual concentration
potassiumdichromate	10 56	11 57
diisopropylamine	10 56 320	9.1 72 315
di(2-ethylhexyl)phtalate	------- *	------- *
2,4-dinitrotoluene	1.0 3.2	0.79 2.4
2,6-dimethylquinoline	0.32 0.56 1.0 1.8	0.44 0.41 1.3 1.2
2,4-dichloroaniline	0.10 0.32 1.0 3.2 5.6	0.10 0.50 0.87 2.9 4.7
tricresylphosphate	------- *	------- *
tetrapropylenebenzene-sulphonate	10	9.4

* analyses failed

Table 6. Comparison of the 96-h LC50-values (in mg/l, nominal) obtained from short term toxicity tests among five fish species.

Compound	G. aculeatus	B. rerio	J. floridae	O. latipes	P. reticulata
potassiumdichromate	85	170	250	160	230
diisopropylamine	840	620	1600	480	> 1000
di(2-ethylhexyl)-phtalate	> 0.32	> 0.32	> 0.32	> 0.32	> 0.32
2,4-dinitrotoluene	6.4	> 16	> 16 < 32	> 16	> 16
2,6-dimethyl-quinoline	13	8.0	11	13	7.5
2,4-dichloro-aniline	10	9.0	8.7	20	22
tricresylphosphate	0.51	> 1.0	5.0	4.9	5.5
tetrapropylene-benzenesulphonate	13	17	20	16	20

Table 7. Comparison of the lowest 96-h NOEC[(*)]-values (in mg/l, nominal) obtained from short term toxicity tests among five fish species.

Compound	G. aculeatus	B. rerio	J. floridae	O. latipes	P. reticulata
potassiumdichromate	32	74	56	32	56
diisopropylamine	320	130	100	180	100
di(2-ethylhexyl)-phtalate	≥ 0.32	≥ 0.32	≥ 0.32	≥ 0.32	≥ 0.32
2,4-dinitrotoluene	1.0	1.8	5.6	1.8	1.8
2,6-dimethyl-quinoline	0.56	3.2	3.2	1.0	1.0
2,4-dichloro-aniline	1.8	3.2	5.6	3.2	5.6
tricresylphosphate	0.18	0.18	1.0	1.8	1.0
tetrapropylene-benzenesulphonate	5.6	10	5.6	5.6	10

(*) E = mortality, abnormal behaviour and appearance

Table 8. Comparison of the lowest NOEC(*)-values (in mg/l, nominal) obtained from long term toxicity tests among five fish species, including early life stage tests with G. aculeatus, B. rerio, J. floridae and O. latipes.

Compound	G. aculeatus	B. rerio	J. floridae	O. latipes	P. reticulata
potassiumdichromate	18	10	3.2	10	10
diisopropylamine	180	56	32	100	32
di(2-ethylhexyl)-phtalate	≥ 0.32	≥ 0.32	≥ 0.32	≥ 0.32	≥ 0.32
2,4-dinitrotoluene	1.0	1.0	1.0	1.0	3.2
2,6-dimethyl-quinoline	1.0	0.56	1.0	1.0	3.2
2,4-dichloro-aniline	0.32	0.56	0.32	0.32	1.0
tricresylphosphate	0.00032	0.0056	0.01	0.01	1.0
tetrapropylene-benzenesulphonate	1.0	5.6	3.2	3.2	10

(*) E = mortality, sublethal effects during embryonic development or growth

Table 9. Comparison of the NOEC(*)-values of the embryonic stage (in mg/l, nominal) obtained from early life stage tests with four egg-laying fish species.

Compound	*G. aculeatus*	*B. rerio*	*J. floridae*	*O. latipes*
potassiumdichromate	≥ 100	≥ 180	≥ 1000	≥ 100
diisopropylamine	560	320	1000	320
di(2-ethylhexyl)-phtalate	≥ 0.32	≥ 0.32	≥ 0.32	≥ 0.32
2,4-dinitrotoluene	3.2	≥ 10	10	1.0
2,6-dimethyl-quinoline	1.0	1.0	3.2	1.0
2,4-dichloro-aniline	1.8	3.2	3.2	3.2
tricresylphosphate	0.0032	0.32	1.0	0.032
tetrapropylene-benzenesulphonate	10	10	10	10

(*) E = mortality, sublethal effects during embryonic development and delayed time of hatching

APPENDIX A

Rearing of G. aculeatus, the three-spined stickleback

G. aculeatus is being reared under standardized conditions. They are kept in glass containers at a temperature of 19 ± 1 C and long-day conditions (16L and 8D). Adult female specimens are kept in 100-l aquaria (35 specimens per aquarium). The males which are used for breeding are kept in 20-l aquaria (one fish per aquarium). Visual contact between the males has to be avoided because of possible stress.

The animals are reared in Dutch Standard Water (DSW; specifications see appendix b). The sediment in the aquaria consists of a mixture of pebbles and clay. To avoid stress, some Vallisneria is cultured concurrently in the aquaria.

The water from each aquarium is filtered constantly (carbon and perlon filters) and aerated. The levels of pH, NO_2 and total hardness (all checked weekly) are kept between 6.5 and 8.4, < 0.1 mg/l and between 10 and 14 ^{0}DH respectively. The temperature is checked daily and is kept between 18-20 ^{0}C. The adult males and females are fed with Tetramin (dry food, Tetrawerke, W. Germany) four times a day and with deep-frozen adult artemia's once a day. The sexual mature males which are used for breeding are fed once a day with deep-frozen adult artemia's and if available with daphnids or one day old guppies.

When the male reaches sexual maturity (this is indicated by the characteristic pattern of body-coloration) he builds a nest. To build a nest, the stickleback is supplied with a lot of strains of flax (length of 5 cm). The sexually mature females can be recognized because of the the fact that the ventral side of the body is swallen when eggs are present. Placing a gravid female to a sexual mature male at this stage, usually results in spawning and fertilization of the eggs in the nest. The number of eggs varies from 30-200 depending of age and size of the female.

The eggs are separated from each other under a microscope (magnification 12x) and rinsed in a diluted solution of formaldehyde (0.04%) for about a minute to prevent the eggs from moulding. The eggs (max. 200) are placed in small glass containers with 2 l of DSW; this medium has been renewed 3 times a week.

Hatching of the young fish can be expected after approximately 7 days at a temperature between 18-20 C. The young fish are kept in 20-1 aquaria (200 specimens per aquarium). After 4 weeks, the sticklebacks are transfered into large aquaria (100 l) in which they stay until they reach maturity (after approximately 5 months). After hatching the fish are being fed with Brachionus rubens for 3 days, the next two weeks with Artemia nauplien and then up to 4 weeks after hatching Artemia nauplien and once a day Tetramin.

APPENDIX B

Composition and preparation of DSW (fresh water)

Composition stock solution I :

1 $NaHCO_3$	50 g	Merck 6329 (purity ≥ 99,5%)
2 $KHCO_3$	10 g	Merck 4854 (purity ≥ 99.5%)
3 distilled water	1 litre	

Dissolve 1 and 2 in 3.
The solution is sterilized by filtration through a membrane filter (0.2 um).

Composition stock solution II :

1 $CaCl_2.2H_2O$	100 g	Merck 2382 (purity ≥ 99.5%)
2 distilled water	1 litre	

Dissolve 1 in 2.
The solution is sterilized by autoclaving (45 min, 120 ^oC).

Composition stock solution III :

1 $MgSO_4.7H_2O$	90 g	Merck 5886 (purity ≥ 99.5%)
2 distilled water	1 liter	

Dissolve 1 in 2.
The solution is sterilized by autoclaving (45 min, 120 ^oC).

Preparation of DSW

Add 100 ml of each stock solution to 50 litre demineralized water.
Then the water has to be aerated for at least one hour before using.
The pH will be 8.4 (calculated). DSW can be prepared in large amounts and keeps its good composition for about one month when aerated thoroughly.