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Effects of Exposing Two Non-Target Crustacean Species, *Asellus aquaticus* L., and *Gammarus fossarum* Koch., to Atrazine and Imidacloprid

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Abstract The physiological responses of two freshwater crustaceans, *Asellus aquaticus* L. and *Gammarus fossarum* Koch., following in vitro exposure to two pesticides were measured. Both species responded to short-term exposure with elevated levels of Respiration (R) and/or lower levels of Electron Transport System (ETS) activity. 1 h exposure to concentrations of up to 10 mg L^{-1} showed an effect in both test species. Laboratory tests confirmed that *G. fossarum* is more sensitive to short-term pesticide exposure than *A. aquaticus*. ETS/R ratio may be used as a quick predictor of effects on organisms exposed to pesticides.

Keywords Pesticide stress assessment · Non-target species

The majority of pesticides are designed to be used in a terrestrial environment, however substantial amounts end up in aquatic ecosystems, in either surface or groundwater (Fernandez-Alba et al. 2002). Their effects on aquatic ecosystems may arise from chronic exposure (long-term and low concentrations), as well as from short-term exposure to high concentrations that can result from accidents, improper use, or run-off from treated fields. Non-target animal populations can be affected and some need more than 6 months for their abundance to recover after pesticides run-off that end up in streams (Liess and Schultz 1999). On several occasions it has been suggested that a broader spectrum of aquatic test animals should be used before newer pesticides (like imidacloprid) can be classified as being safer than those currently applied (Munn and Gillom 2001; Jemec et al. 2007). Crustaceans are frequently used as bioindicators in aquatic toxicity tests due to their prolific breeding, high abundance in nature and sensitivity to anthropogenic toxic compounds in water bodies which they inhabit (Fernandez-Alba et al. 2002). Furthermore, the presence of toxic compounds can be found in their tissues long after exposure, thus external influences can be monitored and spotted after incidents. Song et al. (1997) reported that imidacloprid can be used safely with regard to freshwater arthropods, although it was already known that some aquatic arthropods can be even more susceptible to imidacloprid than *D. magna* (Fernandez-Alba et al. 2002; Jemec et al. 2007). The main disadvantage regarding toxicity tests in *Daphnia* is that their reproduction is based on parthenogenesis, which produces genetically identical offspring. Toxicity tests for *D. magna* therefore offer a limited insight into intraspecific responses on toxic substances.

Selected new species–water louse, *Asellus aquaticus* L. and stream scud, *Gammarus fossarum* were chosen for toxicity tests in relation to their differences in habitat preference. Since *D. magna* is a pond/pelagic species, we selected two, which in addition to lentic can also be found in lotic ecosystems. Due to prevailing sexual reproduction their genetic variability is relatively higher than in *D. magna*, therefore they offer better insight into interspecific responses (Sket et al. 2003). The purpose of our study was to test the both species for differences in stress responses as a function of habitat preference.

Materials and Methods

Specimens of *A. aquaticus* were collected from Zadnji kraj on intermittent Lake Cerkniško Jezero (central Slovenia)

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with low or no rural activity. *G. fossarum* was collected from a small permanent karstic spring located near the village Duplje (northern Slovenia). The catchment area has low human activity. Chemical water quality analysis (ion chromatography, alkalinity, pH, oxygen content and saturation) were performed on samples from both sampling sites and showed no pollution. Animals were transported to the laboratory in a cool box. Up to one day prior to experiments test animals were kept in a laboratory at 10°C, with 12/12 h day/night cycle, in water from the sampling location, which was partly (at two-day intervals) replaced by synthetic water (ISO-standard 6341 1996). Animals were fed on biofilm grown on leaves of black alder (*Alnus glutinosa* L.) infected by bacteria and mould.

Sub-lethal toxicity was studied with standard toxicity tests. The effective and lethal dose concentrations, LC_{50} 48 h and EC_{50} 24 h, were determined as the concentration at which 5 animals out of 10 were paralyzed (only respiration movement was left) (EC_{50} 24 h) or died (no movement at all) (LC_{50} 48 h) (Clesceri et al. 1998). Another set of test animals were later exposed for 1 h (Cold and Forbes 2004) to the same concentrations of two selected toxic compounds prior to respiration measurements.

Atrazine (i.e., 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) was obtained from Riedel-de Haën, 35702 Pestnal® as analytical standard of technical grade 99.9% ($M = 215.69$ g mol^{-1}). A stock solution was prepared at a concentration of 100 mg L^{-1} in methanol (technical grade (GC) = 99.8%), thus the initial concentration for the first exposure solution did not exceed 0.1% of methanol. For the wide range finding toxicity test (WRFTT) nominal test concentrations of 0.01, 0.1, 1.0, 10 and 100 mg L^{-1} of atrazine were used. The highest concentration used, 100 mg L^{-1}, contained 0.1% of methanol. A negative control solution containing 0.1% of methanol was used to check for mortality caused by solvent phase. For the definitive acute toxicity test (DATT), nominal concentrations 0.3, 1, 3, 10 and 30 mg L^{-1} of atrazine were used. The numbers of animals affected by each concentration and their mortality was monitored every 6–12 h, from which effective and lethal concentrations were calculated. Selected 1 h exposure concentrations for *A. aquaticus* were 5 and 10 mg L^{-1} and, for *G. fossarum*, 1, 3 and 10 mg L^{-1} of atrazine (Tables 2, 3).

Stock solutions of imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazol idinimine), as the original Confidor SL 200, were prepared in bi-distilled water containing 200 g L^{-1} of imidacloprid. This aqueous soluble concentrate was obtained from Pinus d.d. (Bayer CS d.o.o., Slovenia) and stored at 4°C.

For WRFTT, nominal test concentrations 0.01, 0.1, 1, 10 and 100 mg L^{-1}, while for DATT they were 1, 3, 10, 30, 100 mg L^{-1} of imidacloprid. Effective and lethal concentrations were calculated from data gained by monitoring the effects every 6–12 h. Selected 1 h exposure concentrations for both test species were 0.01, 0.1, 1.0 and 10 mg L^{-1} of imidacloprid (Tables 4, 5).

Prior to the experiment, animals were kept in synthetic water at 10°C. After 24 h of fasting, animals were exposed to water containing selected concentration of pesticides for 1 h according to Cold and Forbes (2004). Animals were transferred to pesticide-free and oxygen-rich water immediately after termination of exposure to pesticides. For the respiration measurements the test chambers were completely darkened. A control experiment was run with a set of animals that was not exposed to pesticide, but for 1 h to synthetic water only (in the results referred to as Control).

Respiration was measured with a microrespirometer Presens OXY-4 oxygen meter (PreSens GmbH, Regensburg, Germany) with polymer optical fibres inserted airtight into three flow-through test chambers, positioned parallel to each other. The microrespirometer consists of a water tank with aerated (=air saturated) water. This was connected with a Viton tube to a flow-through test chamber with oxygen sensor measuring the concentration of oxygen entering the chamber. After the chamber, a tube connection splits into three parallel tubes, each connected to a test chamber (5 mm in diameter and 25 mm long glass tubes) containing an individual test animal and equipped at the outflow by another oxygen sensor. A peristaltic pump on the end of the system creates negative pressure to produce a flow of water out of all three test chambers (with approx. rate of 5 mL h^{-1}). The oxygen concentrations on entering and leaving each test chamber were recorded on-line by PC (at five second intervals) and the drop of oxygen concentration was recalculated for each test chamber separately. Respiration was measured in the water reservoir at a constant temperature of 10.0 ± 0.1°C.

ETS activity was measured using the method designed by Packard in 1971 and improved by G-Tóth in 1993, followed exact details on the method as described by Simčič and Brancelj (2003).

OpenOffice Calc was used for sorting and calculating primary data from all experiments. Data sets from each experiment were analyzed in computer programs Sigma Stat 3.5 (SYSTAT) and JMP 7 (SAS). For ETS/R ratios at the start, basic descriptive statistics were extracted; if normality test and equal variance test passed, ANOVA or MANOVA tests were performed. When normality tests failed, nonparametric tests on ranks were used (Kruskal–Wallis One Way Analysis of Variance, Mann–Whitney Rank Sum Test). Additional tests (Dunn's Method and Holm–Sidak method) were performed in order to establish differences between groups that were on the margin of significance.

Results and Discussion

LC_{50} (48 h) and EC_{50} (24 h) for atrazine are compared in Table 1 for *A. aquaticus* and *G. fossarum*. Respiration (R) in *A. aquaticus* shows that animals in group 3 (exposed to 10 mg L^{-1} atrazine for 1 h) have significantly higher R (ca. 2.5-fold) than the control (ANOVA, $p < 0.001$). No statistical changes in ETS activity were observed on 1 h exposure to atrazine (ANOVA, $p > 0.05$) (Table 2). In *G. fossarum* the values of R were significantly higher, by ca. 1.3-fold in groups 3 and 4 (concentrations of 3 and 10 mg L^{-1}, ANOVA, $p < 0.01$), while ETS activity did not change at higher concentrations of atrazine (Table 3). The ETS/R ratio was significantly higher than the control value in *A. aquaticus* in group 3 for 10 mg L^{-1} of atrazine, (ANOVA, $p < 0.001$), but not at lower concentration (5 mg L^{-1} of atrazine) (Fig. 1a). In *G. fossarum* ETS/R ratios from all tested groups exposed to atrazine (1, 3 and 10 mg L^{-1}) differ significantly from those for the control group (ANOVA, $p < 0.05$) (Fig. 1b). The EC_{50} values obtained here for *A. aquaticus* at 17.5 mg L^{-1} and for *G. fossarum* at 6 mg L^{-1}, (24 h, at 10°C) are comparable to those reported by Munn and Gillom (2001) who defined atrazine effective concentrations EC_{50} (24 h) for *Gammarus pulex, G. italicus, Daphnia pulex* and *Hyalea azteca* of 14.9, 10.1, 41.5, 14.7 mg L^{-1}, respectively. Although the temperature at which their experiments were performed was not quoted. Our values of LC_{50} (48 h) were 42.5 mg L^{-1} of atrazine for *A. aquaticus* and 7.5 mg L^{-1} of atrazine for *G. fossarum*, which can be compared with those of Pantani et al. (1997) for LC_{50} (96 h), 10.1 mg L^{-1} of atrazine for *Gammarus italicus* and 3.3 mg L^{-1} of atrazine for *Echinogammaus tibaldii*. The reported time intervals, (96 h), make it difficult to compare and evaluate these results with our LC_{50} (48 h), but the concentrations are of a similar order of magnitude.

Acute 1 h exposure of *A. aquaticus* to atrazine at a concentration of 10 mg L^{-1} (group 3) resulted in significantly higher R but similar ETS activity, leading to a significantly lower ETS/R ratio (Table 2; Fig. 1a). No significant differences in R and ETS/R were observed exposure to lower concentrations (i.e. 5 mg L^{-1} or less). A low ETS/R ratio at high concentrations of pesticides indicates stress conditions in test animals. In *G. fossarum*, the ETS/R ratio for all exposed groups was significantly lower due to higher respiration, indicating higher sensitivity of *G. fossarum* to the pesticide than *A. aquaticus*. Respiration was significantly increased in *G. fossarum* in group 3 (3 mg L^{-1}) and group 4 (10 mg L^{-1}). Short term exposure of both *G. fossarum* and *A. aquaticus* to atrazine did not affect ETS activity, as was also observed in the experiment with imidacloprid (compare 4.2). Values of ETS in the atrazine experiment were similar to those obtained from other experiments where no stress-induced chemicals were used (Simčič and Brancelj 2003; Simčič et al. 2005).

Table 1 Lethal and effective concentrations

		A. aquaticus [mg L^{-1}]	*G. fossarum* [mg L^{-1}]
Atrazine	LC_{50} (48 h)	42.5	7.5
	EC_{50} (24 h)	17.5	6
Imidacloprid	LC_{50} (48 h)	8.5	0.8
	EC_{50} (24 h)	0.8	0.07

Table 2 R and ETS activity in specimens of *Asellus aquaticus* exposed for 1 h to different concentrations of atrazine

Treatment	Group 1		Group 2		Group 3	
	Control	SD	5 mg L^{-1}	SD	10 mg L^{-1}	SD
N	9		10		9	
WW (mg)	11.8	3.3	9.9	3.4	13.7	3.9
R (μL O_2 mg^{-1} h^{-1})	0.054	0.016	0.056	0.021	0.133***	0.034
ETS (μL O_2 mg^{-1} h^{-1})	0.478	0.096	0.468	0.060	0.526	0.063

*** $p < 0.001$

Table 3 R and ETS activity in specimens of *Gammarus fossarum* exposed for 1 h to different concentrations of atrazine

Treatment	Group 1		Group 2		Group 3		Group 4	
	Control	SD	1 mg L^{-1}	SD	3 mg L^{-1}	SD	10 mg L^{-1}	SD
N	18		21		22		18	
WW (mg)	16.3	3.5	14.8	3.4	16.2	3.8	14.1	4.2
R (μL O_2 mg^{-1} h^{-1})	0.074	0.018	0.087	0.024	0.097**	0.032	0.094**	0.018
ETS (μL O_2 mg^{-1} h^{-1})	0.478	0.065	0.424	0.094	0.435	0.073	0.458	0.092

** $p < 0.01$

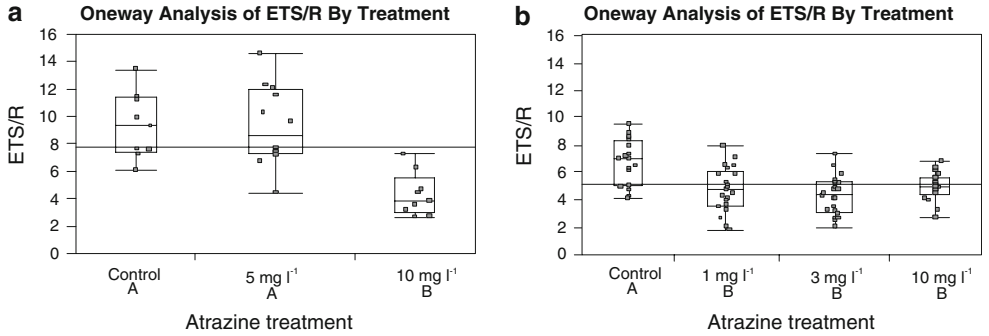

Fig. 1 Box and Whiskers plots of ETS/R ratio for *Asellus aquaticus* (Fig. 1a) and *Gammarus fossarum* (Fig. 1b) exposed for 1 h to various atrazine concentrations at 10°C. Dots indicate results from individual animals, boxes indicate mean ± 1st quartile, and the horizontal line the mean of all the groups. On the *x axis* is treatment with different concentrations of atrazine, (1a: n = 9; 10; 9 and 1b: n = 18; 21; 22; 18). Results of pair-wise comparisons are indicated below the graph, the different letter below data groups meaning that groups differ significantly ($p < 0.001$)

A. aquaticus was shown to be less sensitive than *G. fossarum*, based on the higher lethal and effective dose concentrations for the former, assuming that the animals were taken from a "pesticide free" environment. Moreover, the larger decrease in ETS/R values in *G. fossarum* is also in accordance with reports of other authors like Graça et al. (1994) and Maltby (1995) who found that *Gammarus* species were more sensitive than *A. aquaticus*.

LC_{50} (48 h) and EC_{50} (24 h) for imidacloprid exposure of *A. aquaticus* and *G. fossarum* are in Table 1. Respiration (R) in *A. aquaticus* exposed to high short-term concentrations of imidacloprid (1 and 10 mg L^{-1}) was significantly higher by ca. 1.2-fold, compared to the control (ANOVA, $p < 0.01$). In contrast to R, ETS activity values in all groups were similar, except in group 5 where it was 1.4-fold lower (exposed to 10 mg L^{-1}, ANOVA, $p < 0.001$) (Table 4).

In *G. fossarum* the values of R and ETS did not differ significantly for most of the groups, except for ETS (1.4-fold) in group 4 (10 mg L^{-1}, Kruskal–Wallis, $H = 19.721$, $p < 0.05$) (Table 5). Higher concentrations of imidacloprid are correlated with lower mean ETS/R ratio for both *A. aquaticus* (Kruskal–Wallis, $H = 51.053$, $p < 0.05$) (Fig. 2a) and *G. fossarum* (ANOVA, $p < 0.001$) (Fig. 2b). Effective concentrations (EC_{50} (24 h)) determined for our test animals (0.8 mg L^{-1} for *A. aquaticus* and 0.07 mg L^{-1} for *G. fossarum*) for imidacloprid are approximately one magnitude greater for *A. aquaticus* than for *G. fossarum*, the value for the latter being in agreement with that reported by Kreutzweiser et al. (2007). These authors also found high mortality of aquatic insects at a concentration higher than 0.13 mg L^{-1}, and significant inhibition of feeding at concentrations above 0.012 mg L^{-1} for imidacloprid at 20 ± 3°C. LC_{50} (48 h) concentrations in the more tolerant *A. aquaticus*, which is a common inhabitant of stagnant water where oxygen concentrations can be low, were found to be similar to those reported by Song et al. (1997) for *Daphnia magna.* The latter authors determined the acute toxicity test concentration of LC_{50} (48 h) at 27°C to be 10.4 mg L^{-1}, i.e. one magnitude higher than in the more sensitive *G. fossarum* (1 mg L^{-1}), which is a typical inhabitant of running water, rich in oxygen. The reported concentrations could not be directly compared with that for *Artemia* sp. (LC_{50} (48 h) = 361.2 mg L^{-1}), which is a common inhabitant of hypersaline salt ponds. At lower temperature (20°C) the effects were tested only for *Daphnia magna* (Song et al. 1997) and are higher, the LC_{50} (48 h) concentration being 17.4 mg L^{-1}. However, our LC_{50} (48 h) values, measured at 10°C, are lower than those reported by Song et al. (1997) and Sánchez-Bayo and Goka (2006) who

Table 4 R and ETS activity in specimens of *Asellus aquaticus* exposed for 1 h to different concentrations of imidacloprid

	Group 1		Group 2		Group 3		Group 4		Group 5	
Treatment	Control	SD	0.01 mg L^{-1}	SD	0.1 mg L^{-1}	SD	1 mg L^{-1}	SD	10 mg L^{-1}	SD
N	9		24		30		26		30	
WW (mg)	15.7	3.0	21.8	4.1	16.9	2.8	22.3	4.3	19.7	2.9
R (µL O_2 mg^{-1} h^{-1})	0.141	0.044	0.148	0.033	0.131	0.030	0.192**	0.044	0.169**	0.031
ETS (µL O_2 mg^{-1} h^{-1})	0.296	0.042	0.291	0.046	0.273	0.045	0.281	0.047	0.208***	0.031

** $p < 0.01$

*** $p < 0.001$

Table 5 R and ETS activity in specimens of *Gammarus fossarum* exposed for 1 h to different concentrations of imidacloprid

	Group 1		Group 2		Group 3		Group 4	
Treatment	Control	SD	0.01 mg L^{-1}	SD	0.1 mg L^{-1}	SD	10 mg L^{-1}	SD
N	11		25		21		18	
WW (mg)	14.4	4.3	22.7	3.9	22.3	4.5	15.8	3.2
R (μL O_2 mg^{-1} h^{-1})	0.089	0.021	0.102	0.034	0.133	0.032	0.113	0.039
ETS (μL O_2 mg^{-1} h^{-1})	0.396	0.104	0.354	0.069	0.353	0.082	0.271**	0.071

** $p < 0.01$

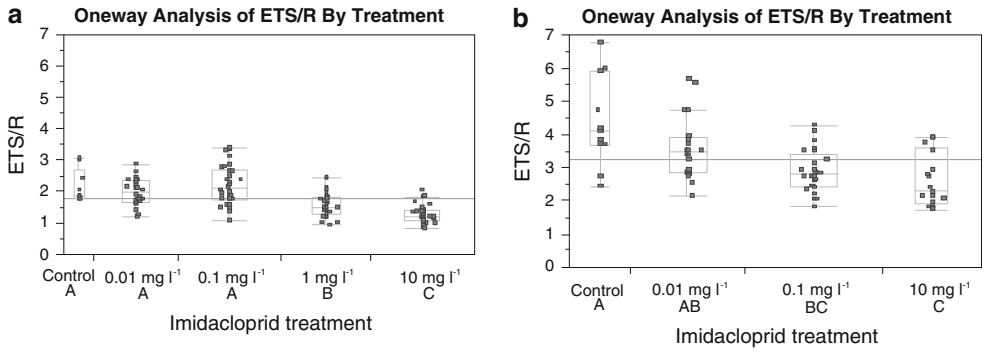

Fig. 2 Box and Whiskers plots for ETS/R ratio for *Asellus aquaticus* (2a) and *Gammarus fossarum* (2b) exposed for 1 h to different imidacloprid sublethal concentrations at 10°C. Dots indicate the results from individual animals, boxes indicate means ± 1st quartile, and the horizontal line is the mean of all the groups. On the *x axis* are treatments with different concentrations of imidacloprid (2a: n = 9; 24; 30; 26; 30 and 2b: n = 11; 25; 21; 18). Results of pair-wise comparisons are indicated below the graph, the different letter below data group means groups differ significantly ($p < 0.001$)

obtained values from 65 up to 133 mg L^{-1} for *Daphnia* sp. Jemec et al. (2007) determined LC_{50} (48 h) (as LOLC–lowest observed lethal concentration) for *D. magna* of 10 mg L^{-1} for imidacloprid (product Confidor SL 200), which is the same as LC_{50} (48 h) for our less susceptible species *A. aquaticus.*

All these comparisons of values for different animals as well as for the same animals indicate that correct experimental temperature was selected and that results can be compared to results from other authors. The significant decrease in ETS/R ratio in *A. aquaticus* after 1 h exposure in group 4 (1 mg L^{-1}) and group 5 (10 mg L^{-1} of imidacloprid) (Fig. 2a) was the result of a combination of higher respiration and lower ETS activity than those for the control (Table 4). Thus, imidacloprid influences not only respiration but also ETS activity. Choi et al. (2001) reported a similar decrease of ETS activity in *Chironomus riparius* exposed to high concentration of fenitrothion. This effect is a consequence of different processes, including oxidative stress. Glutathione peroxidase activity was decreased and, since the enzyme is involved in the reduction of lipid hydroperoxide, a decrease of its activity may enhance the peroxidation of cells and membranes. Partial damage to the inner mitochondria membrane by lipid peroxidation may impair the function of ETS and reduce its activity which took place in mitochondrial membranes only. We found similar effects of pesticides on enzymatic activity in *G. fossarum*. The lower ETS/R ratio in groups 3 and 4 in *G. fossarum* (0.1 mg L^{-1} and 10 mg L^{-1} of imidacloprid) is due to reduction of ETS activity, which was significantly lower in group 4 (10 mg L^{-1} of imidacloprid) and slightly decreased in the other two exposed groups 2 and 3 (Table 5; Fig. 2b). Respiration values for both species stayed relatively unchanged compared to control in groups exposed to lower concentrations but were significantly higher in *A. aquaticus* exposed to higher concentrations (1 and 10 mg L^{-1}). At the same time, even short exposure to high concentrations (10 mg L^{-1}) partly inactivates/destroys the ETS in both tested species. The results with imidacloprid indicate that *G. fossarum* from running water is more affected by short-term higher concentrations of imidacloprid than is *A. aquaticus,* a common inhabitant of standing water (ponds, lakes). *G. fossarum* reacts with a lower ETS/R, even at very low concentrations of pesticide (i.e. 0.01 mg L^{-1}), while *A. aquaticus* reacts only to concentrations that are at least two orders of magnitudes higher. In both sets of experiments *A. aquaticus* was shown to be less sensitive to atrazine and imidacloprid than *G. fossarum.*

Water louse prefers standing or slow flowing waters, retention time of both pesticides is therefore longer than in fast flowing waters, which stream scud prefers. Therefore water louse developed a relatively higher resistance to allochtonous substances.

This combination of measurements of R and ETS activity, provides a good assessment of stress after exposure of animals to pesticides for short periods of time. Stress in exposed animals is normally shown as a reduction in ETS/R ratio, which drops to a value close to 1 when the animal is highly affected. The ETS/R ratios are high in normal conditions; normally well above 2–4 (as indicated in the control group). Values close to 1 indicate that the animal is using 100% of its respiratory potential and that the whole enzyme system is exploited. Hypothetically, R should increase under stress conditions but ETS should stay at the same level under short exposure times. This would be reflected in decreased ETS/R values. Some pesticides at higher concentrations, not only increase the demand on energy in animals, but actually destroy the energy production system. Those types of pesticide that affect animals on both levels (ETS & R) are thus harmful also for non-target organisms, even at low concentrations. Decrease in ETS activity is reflected in reduced ETS/R ratio, which indicates greater stress on test animals. Such effects of pesticides on a biochemical level could not be detected by standard toxicity tests.

Both tested animals, *A. aquaticus* and *G. fossarum*, are very susceptible to short-term atrazine and imidacloprid exposure. *A. aquaticus* shows significant effects at concentrations of 10 mg L^{-1} or more of atrazine and higher than 1 mg L^{-1} for imidacloprid. The more sensitive *G. fossarum* shows significant effects at concentrations higher than 1 mg L^{-1} of atrazine and higher than 0.1 mg L^{-1} of imidacloprid. Elevated levels of R and diminution of ETS activity result in a lower ETS/R ratio, which we propose in this article to be an indicator of stress. Using this method, maximum permissible levels of toxic compounds in water bodies can be determined more accurately. With such more reliable data, better environmental policies and industrial discharge regulations can be applied.

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