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Oxidative stress in triazine pesticide toxicity: a review of the main biomarker findings

Tanja Živković Semren, Suzana Žunec, and Alica Pizent

Institute for Medical Research and Occupational Health, Zagreb, Croatia

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This review article provides a summary of the studies relying on oxidative stress biomarkers (lipid peroxidation and antioxidant enzymes in particular) to investigate the effects of atrazine and terbuthylazine exposure in experimental animals and humans published since 2010. In general, experimental animals showed that atrazine and terbuthylazine exposure mostly affected their antioxidant defences and, to a lesser extent, lipid peroxidation, but the effects varied by the species, sex, age, herbicide concentration, and duration of exposure. Most of the studies involved aquatic organisms as useful and sensitive bio-indicators of environmental pollution and important part of the food chain. In laboratory mice and rats changes in oxidative stress markers were visible only with exposure to high doses of atrazine. Recently, our group reported that low-dose terbuthylazine could also induce oxidative stress in Wistar rats. It is evident that any experimental assessment of pesticide toxic effects should take into account a combination of several oxidative stress and antioxidant defence biomarkers in various tissues and cell compartments. The identified effects in experimental models should then be complemented and validated by epidemiological studies. This is important if we wish to understand the impact of pesticides on human health and to establish safe limits.

KEY WORDS: *atrazine; antioxidant enzymes; lipid peroxidation; terbuthylazine*

The extensive use of pesticides in agriculture, public health, commerce, and individual households throughout the world speaks a lot about the importance of these compounds, but also raises concern about the risks involved for the environment, wildlife, and human health. Beside direct exposure of individuals who apply pesticides in agricultural, occupational, or residential settings, people are exposed to pesticides and their degradation products indirectly through water, air, dust, and food. These indirect routes generally involve low-level, yet long-term exposure (1). An increasing number of epidemiological studies have been suggesting that current levels of exposure are associated with risks to human health, including chronic diseases, cancers, neurological deficits, birth defects, and reproductive disorders (2, 3).

Pesticides can produce adverse physiological or biological effects, with a variety of biochemical changes at the molecular, cellular, or tissue level. These biochemical changes can be used as biological markers of effects in epidemiological studies as well as in animal toxicology studies. The most common biomarkers used to assess pesticide effects are related to DNA and RNA damage, modulation of gene expression, and oxidative stress (2). Although the underlying mechanisms are not known, a coherent body of evidence indicates that some pesticides, including organophosphates, organochlorines, organofluorines, carbamates, pyrethroids, bipyridyl herbicides, chloroacetanilide herbicides, and triazine herbicides can induce oxidative stress (4-6).

Biomarkers of oxidative stress have been demonstrated to be sensitive enough to high and low pesticide exposure alike. Thanks to oxidative stress biomarkers like malondialdehyde, F2-isoprostanes, thiobarbituric reactive substances, catalase and superoxide dismutase activities, 8-oxo- or 8-OH-deoxyguanosine, molecular epidemiological studies have provided us with evidence that oxidative stress is one of the key links between pesticides toxicity and multi-stage carcinogenesis (7-10). Furthermore, field studies in aquatic organisms confirm that oxidative stress is generated by exposure to several classes of pesticides (organochlorines, organofluorines, organophosphates, carbamates, pyrethroids, bipyridyl herbicides, triazine herbicides, and chloroacetanilide herbicides) (11). Induction of oxidative stress has also been reported in test animals exposed to organophosphorus pesticides, *N*-methyl carbamates, organochlorines, pyrethroids, and triazines (12). However, most animal studies use high concentrations of pesticides that are not normally encountered in the environment.

Oxidative stress occurs when the antioxidant defence mechanisms are overwhelmed by reactive oxygen species (ROS) generated through exposure to pesticides (10). This so called redox imbalance (with excess ROS) can modify proteins and lipids, change the DNA structure, activate stress-induced transcription factors, and produce inflammatory cytokines. General pathways by which pesticides increase ROS levels are their oxidative

Correspondence to: Suzana Žunec, PhD, Toxicology Unit, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, HR-10001 Zagreb, Croatia, E-mail: *suzana@imi.hr*

metabolism by cytochrome P450 enzymes, generation of redox-active metabolites, and impairment of the electron transport cascades in mitochondria (10, 13). ROS react readily with lipids, proteins, carbohydrates, and nucleic acids, and in doing so affect the structure and function of cellular components. When free radicals attack unsaturated fatty acids in cell membranes that contain lipids, lipid peroxidation occurs. One of the most common markers of lipid peroxidation is malondialdehyde (MDA), formed as its product, which is often assayed with thiobarbituric acid (TBA) (14). However, MDA is also a known mutagen (15). Radical chain reaction of lipid peroxidation appears to be a continuous physiological process. This process, if out of control, can alter essential cell functions and lead to cell death (16). Antioxidant defence includes enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and non-enzymatic systems like glutathione (17). SOD is the first step in the defence against oxidative stress, as it catalyses dismutation of two superoxide radicals (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which is then neutralised by the combined action of CAT and GPx in all vertebrates (18). These enzymes have coordinated action, but changes in their levels may tip the balance toward oxidative stress.

As no single biomarker is sensitive and specific enough to identify or predict all oxidative stress effects (19), the aim of this paper was to summarise the representative studies in which the main markers of oxidative stress (20) were found associated with pesticide exposure and to draw conclusions about their significance in effective risk characterisation of pesticides.

We limited our scope to the triazine herbicides, atrazine and terbuthylazine in particular, because they have been well-researched and widely used in the USA and Europe, respectively. Our review includes key published literature and regulatory reports as well as our own results obtained within the project Organic Pollutants in Environment - Markers and Biomarkers of Toxicity (OPENTOX).

Triazine herbicides

Herbicides are the most widely used class of pesticides, accounting for more than 60 % of all pesticides applied in agriculture (21). They aim to inhibit biochemical and physiological processes that regulate plant growth. Their target sites are usually enzymes involved in the primary metabolic pathways or proteins carrying out essential physiological functions in plants (22). The main mechanism of triazine herbicides action is the inhibition of photosynthesis. Triazines bind to the QB protein in the photosystem II reaction centre and block the flow of electrons through the photosynthetic electron transport chain (23). Since they provide application flexibility, triazines are extensively used in conservation tillage programs as part of sustainable agriculture and are very effective in controlling many weeds resistant to other herbicides (24).Today, one or more of the triazine herbicides are marketed in more than 100 countries around the world (24). Although several classes of triazine herbicides have been developed since the 1950s, chloro-s-triazines have been a mainstay of preemergent application.

Evaluation of triazine herbicides revealed that these are chemicals with low toxic potential for humans; however, there are many controversies on this issue. For instance, in the beginning of the 2000s science and regulatory reviews conducted by the United Kingdom for the European Union (25-27), the Australian Pesticides and Veterinary Medicines Authority (28, 29), the International Agency for Research on Cancer (30), and the US Environmental Protection Agency (EPA) (31, 32) all supported the safety and continued availability of atrazine for weed control. Atrazine is still the most prevalent corn crop pesticide in the US, but in the EU it has been banned since 2004. The European Commission decided to ban atrazine not because of any specific toxicological reasons but because it was concerned that its residues in groundwater might exceed its nominal limit of 0.1 $\mu g\ L^{-1}$ (33). New studies about atrazine's toxicological profile (acute and chronic effects, reproductive and developmental toxicity, mutagenicity, immunotoxicity) and environmental risks brought changes in regulatory positions. According to Dikshith (34), atrazine and associated compounds are of particular concern for human health and the environment due to: 1) evidence of carcinogenicity, reproductive/developmental effects, organ toxicity, immune system effects, and genotoxicity to humans; 2) likely adverse effects on non-target plants and animals, especially on the reproductive and developmental function in aquatic and amphibious species; and 3) extensive contamination of surface, ground, and drinking water.

INVOLVEMENT OF OXIDATIVE STRESS IN ATRAZINE TOXICITY

Atrazine [(6-chloro-*N*-ethyl-N0-(1-methylethyl)-1,3,5-triazine-2,4-diamine] has been extensively studied and reviewed because of its moderate persistence in the environment and contamination of surface, ground, and drinking water that has raised a number of health concerns (35-38). Atrazine is generally considered an endocrine disruptor with adverse effects on the endocrine, central nervous, and immune system (39). Even though some disagree that it disrupts the endocrine system, a number of adverse effects have been reported, especially on the reproductive systems of rats, pigs, fish, and amphibians (39). Atrazine has been reported to cause mammary gland tumours in female Sprague-Dawley rats, which implies that it may be carcinogenic (40). Case-control studies, however, showed only weak associations between atrazine and non-Hodgkin lymphoma, ovarian, and prostate cancer (41-44). Based on the available animal and human data, the International Agency for Research on Cancer (IARC) (30) and the US EPA (31) have characterised atrazine as "not likely to be carcinogenic in humans".

However, the atrazine effects in humans are not fully understood. There are ongoing studies looking for molecular evidence of atrazine's adverse effects, especially at environmental levels. Yet, there is also a coherent body of evidence that atrazine induced oxidative stress. Table 1 summarises the findings of the studies published from 2010 till now, which evidence changes in the main oxidative stress biomarkers.

We start with aquatic organisms first, as they have proven their usefulness as experimental models for the evaluation of adverse effects of atrazine. Tested atrazine concentrations ranged from 0.3 μg L^{-1}, which is the highest environmental concentration reported in Czech rivers (45, 46, 54) and 4.28 μg L^{-1}, which is 1/5 of the 96-hour LC_{50} for common carp (*Cyprinus carpio*) (49, 50), to concentrations as high as hundred or thousand μg L^{-1}. Atrazine did not have any influence on the levels of lipid peroxidation products in aquatic organisms at early stages of development (45-48). In adult aquatic organisms, increases in MDA or TBARS were reported after sub-chronic exposure (14-40 days) to higher atrazine concentrations (≥42.8 μg L^{-1}) (49-52, 54), whereas acute exposure of one or two days did not cause lipid peroxidation (56, 62). An exception is the study by Owolabi et al. (60), who reported higher MDA concentrations after a four-day atrazine exposure.

As for atrazine effects on antioxidant enzyme activities, sub-chronic exposure to an environmentally relevant concentration of atrazine (0.3 μg L^{-1}) caused an increase in the activity of CAT, GPx, GST, and SOD in common carp embryos and larvae, whereas sub-chronic exposure to environmentally relevant concentration of atrazine-2-hydroxy (0.66 μg L^{-1}) did not affect their activities (46). In adult carp, several studies reported that a 40-day exposure to atrazine in concentrations above 42.8 μg L^{-1} resulted in a decrease in CAT, GPx, and SOD activities (49-51).

In adult zebrafish (*Danio rerio*), a 28-day exposure to low atrazine concentrations, including the environmentally relevant 0.3 μg L^{-1} caused a decrease in CAT (54), but exposure to higher concentrations of atrazine (>100 μg L^{-1}) for 14 or 21 days resulted in an increase in CAT and SOD activities in the liver homogenate (52, 53).

In other fish species, sub-chronic exposure to low atrazine increased CAT and SOD activities (56, 59), whereas acute exposure generally lowered CAT, GPx, and SOD activities (55, 57, 58), with exception of neotropical fish, which showed no change (56). Furthermore, in two studies atrazine exposure of *Gammarus kischineffensis* and shrimp *Palaemonetes argentinus* resulted in lower CAT but higher SOD activity (61, 62).

Two studies in drosophilids observed developmental and genotoxic effects (81, 82), which prompted Figuera et al. (63) to investigate if these effects were owed to oxidative stress. To do that, they exposed the embryos (newly fertilised eggs) of the fruit fly *Drosophila melanogaster* to atrazine concentrations ranging between 10 and 100 μmol L^{-1} through diet until they developed into adult flies. While the larvae showed no changes in ROS levels, the adult flies did, so the authors concluded that redox imbalance must have been related to changes in metabolism after metamorphosis. Generally, female flies were more sensitive to oxidative stress; they showed increased ROS levels and antioxidant capacity against peroxyl radicals (ACAP) at atrazine concentration of 10 μmol L^{-1}, while LPO was did not increase until 100 μmol L^{-1}.

The growing evidence of recent declines in pollinator populations has raised great concern about economic and environmental consequences of these declines. One outstanding threat to honeybees (*Apis mellifera*) is the non-target toxicity of pesticides. Honeybees and other pollinators are exposed to atrazine and other herbicides through pollen, nectar, water, and dust, particularly in the vast areas with herbicide-treated monoculture crops. One study (64) investigated lipid peroxidation in caged honeybees exposed to syrup spiked with realistic environmental doses of atrazine (1.25 to 5 ng^{-1} per bee) and found no significant changes in their TBARS levels. In another study (65), honeybees in a laboratory were exposed to atrazine concentrations ranging from 0.1 to 10 μg L^{-1} for 24 hours, whereas hives in the field were treated with 10 μg L^{-1} of atrazine a day for 28 days. GPx and GST activities dropped, whereas MDA levels increased in both the laboratory and hive honeybees.

Rodent studies have made a big contribution to the knowledge about the involvement of oxidative stress in the toxicity mechanisms of atrazine. Table 1 sums up the findings of several important studies on mice and rats, which reported changes in the main oxidative stress biomarkers. For instance, mice receiving 78.25 mg kg^{-1} bw (1/8 LD_{50}) of atrazine intraperitoneally every other day for 14 days had higher liver MDA and GST and lower GSH, antioxidant power (FRAP), SOD, and CAT. The latter two were also low in the kidney tissue (66). Similar effects were noticed in mice receiving 100, 200, or 400 mg kg^{-1} bw of atrazine by gavage every day for 21 days (67).

In the studies conducted on rats, the animals were exposed to atrazine acutely (7 to 14 days) or subchronically (15 to 52 days). The maximum tolerated dose of atrazine in rats, according to Singh et al. (68) and Narotsky et al. (83) is 300 mg kg^{-1} bw or 1/10 of its oral LD_{50}. In a number of studies (summarised in Table 1), sub-chronic exposure to 300 mg kg^{-1} of atrazine resulted in higher MDA levels in the liver and adrenal cortex of rats (70, 72, 79). Higher MDA was also detected in the testes, epididymis, brain, liver, and kidney of rats exposed to 25-200 mg kg^{-1} of atrazine (69, 73, 76, 80). As for the antioxidant defences in rats, higher atrazine doses (300 mg kg^{-1}) increased the activities of CAT, SOD, GPX, and GST and decreased GSH levels in blood and liver (68, 70, 72), whereas all of these markers dropped in the adrenal cortex (79). At doses from 25 to 200 mg kg^{-1}, atrazine lowered CAT activity in rat testis, epididymis, brain, liver, and kidney (69, 71, 73, 76, 80).

In contrast, oral exposure to low atrazine doses (0.3 mg kg^{-1} or 12.5 mg kg^{-1}) did not change MDA in rat liver, kidney, and testis (74, 75) or disturb antioxidant defences by changing

Table 1 *Changes in oxidative stress markers in experimental models after exposure to atrazine*

Experimental model	Route of administration	Exposure duration	Concentration / dose	Oxidative stress markers	Ref.
Fish – common carp, embryos and larvae	water	36 days	0.66 μg L^{-1} (Atrazine 2-hydroxy)	●CAT ●GR ●GSH ●GST ●SOD ●TBARS	Velisek et al. (45)
Fish – common carp, embryos and larvae	water	33 days	0.3 μg L^{-1} 30 μg L^{-1} 100 μg L^{-1} 300 μg L^{-1}	↑CAT (0.3 μg L^{-1}) ↑GPx ●GR ↑GST (0.3 μg L^{-1}) ↑SOD (0.3 μg L^{-1}) ↓SOD (300 μg L^{-1}) ●TBARS	Chromcova et al. (46)
Marbled crayfish eggs	water	77 days	0.66 μg L^{-1} (Atrazine 2-hydroxy)	↑CAT ●GR ●SOD ●TBARS	Velisek et al. (47)
Fish – zebrafish embryos	water	4 days	0.1 mmol L^{-1}	●CAT ●GPx ↓GSH ●TBARS	Adeyemi et al. (48)
Fish – common carp	water	40 days	4.28 μg L^{-1} 42.8 μg L^{-1} 428 μg L^{-1}	↑CAT (4.28 μg L^{-1}-liver and gill) ↓CAT (42.8, 428 μg L^{-1}-liver; 428 μg L^{-1}-gill) ↓GPx (liver; 42.8, 428 μg L^{-1}-gill) ↓SOD (42.8, 428 μg L^{-1} -liver and gill) ↑MDA (42.8, 428 μg L^{-1} -liver; 428 μg L^{-1}-gill)	Xing et al. (49)
Fish – common carp	water	40 days	4.28 μg L^{-1} 42.8 μg L^{-1} 428 μg L^{-1}	↓CAT (428 μg L^{-1} -brain and kidney) ↑GPx (428 μg L^{-1}-kidney) ↓GPx (42.8, 428 μg L^{-1}-brain; 428 μg L^{-1}-kidney) ↓SOD (42.8, 428 μg L^{-1} -brain and kidney) ↑MDA (428 μg L^{-1}-brain; 42.8, 428 μg L^{-1}-kidney)	Xing et al. (50)
Fish – common carp	water	40 days	4.28 μg L^{-1} 42.8 μg L^{-1} 428 μg L^{-1}	↓SOD (42.8, 428 μg L^{-1}-spleen, head, kidney) ↑MDA (42.8, 428 μg L^{-1}-spleen; 428 μg L^{-1}-head kidney)	Wang et al. (51)

Experimental model	Route of administration	Exposure duration	Concentration / dose	Oxidative stress markers	Ref.
Adult female fish zebrafish	water	14 days	1 μg L^{-1} 10 μg L^{-1} 100 μg L^{-1} 1000 μg L^{-1}	↑CAT (1000 μg L^{-1}-liver: 10 μg L^{-1}-ovary) ↓GSH (10, 100, 1000 μg L^{-1} -liver; 1,100, 1000 μg L^{-1} -ovary) ↑SOD (100, 1000 μg L^{-1} -liver; 10 μg L^{-1}-ovary) ↑MDA (100, 1000 μg L^{-1} -liver)	Jin et al. (52)
Fish – zebrafish	water	21 days	2500 μg L^{-1} 5000 μg L^{-1} 10 000 μg L^{-1}	↑CAT (5000 μg L^{-1}) ↑SOD (2500 μg L^{-1})	Zhu et al. (53)
Fish – zebrafish	water	28 days	0.3 μg L^{-1} 3 μg L^{-1} 30 μg L^{-1} 90 μg L^{-1}	↓CAT ↑GPx (30 μg L^{-1}) ↑GR (0.3, 30 μg L^{-1}) ↓GST (90 μg L^{-1}) ●SOD ↑TBARS (30, 90 μg L^{-1})	Blahová et al. (54)
Fish – zebrafish	water	1 day	300 μg L^{-1}	↓CAT (liver) ↓GPx (liver, brain) ↑GSH (kidney) ↓SOD (kidney) ●MDA (liver, kidney, brain)	Shukla et al. (55)
Neotropical fish (*Prochilodus lineatus*)	water	Acute (2 days) and subchronic (14 days)	2 μg L^{-1} 10 μg L^{-1} 25 μg L^{-1}	**Subchronic** ↑CAT (10 μg L^{-1}) ●GSH ●GPx ↑ GST (10 μg L^{-1}) ↑SOD (10 μg L^{-1}) ↑LPO (10 μg L^{-1})	Paulino et al. (56)
Neotropical fish (*Prochilodus lineatus*)	water	1 and 2 days	2 μg L^{-1} 10 μg L^{-1}	↓CAT ↓GPx ↑GR ↓GSH (2nd day) ↓SOD ↓MDA (1st day; 2 μg L^{-1}-2nd day) ↓ROS (1st day; 10 μg L^{-1}-2nd day)	Santos et al. (57)
Neotropical catfish (*Rhamdia quelen*)	water	4 days	2 μg L^{-1} 10 μg L^{-1} 100 μg L^{-1}	↓CAT ↓GPx ↓GR ↓GSH (100 μg L^{-1}) ↓GST ●SOD ● LPO	Mela et al. (58)
Fish – *Channa punctatus* (Bloch)	water	15 days	4238 μg L^{-1} 5300 μg L^{-1} 10600 μg L^{-1}	↑CAT (till day 7) ↓CAT (after day 7) ↑GR (after day 10) ↑SOD ↑TBARS	Nwani et al. (59)

Experimental model	Route of administration	Exposure duration	Concentration / dose	Oxidative stress markers	Ref.
African catfish (*Clarias gariepinus*)	water	Acute (4 days) and Chronic (28 days)	Acute (4 days) 28 µg L^{-1} 30 µg L^{-1} 32 µg L^{-1} 34 µg L^{-1} Chronic (28 days) 7 µg L^{-1} 7.50 µg L^{-1} 8 µg L^{-1} 8.50 µg L^{-1}	**Acute** ↓SOD (28, 30, 34 µg L^{-1}-blood) ↑SOD (gill and liver) ↑MDA (30, 32 µg L^{-1}-blood; gill and liver) **Chronic** ↓SOD (7, 7.5, 8 µg L^{-1} -blood; 7.5, 8, 8.5 µg L^{-1}-gill; liver) ↑SOD (8.5 µg L^{-1}-blood) ↑MDA (7.5, 8 µg L^{-1} -blood; gill and liver)	Owolabi et al. (60)
Gammarus kischineffensis	water	1, 2, 3, 4 days	1000 – 50,000 µg L^{-1}	↓CAT (after 3 days) ●CAT (after 4 days) ↑GST ↓GR ↑SOD	Demirci et al. (61)
Shrimp (*Palaemonetes argentinus*)	water	1 day	400 µg L^{-1}	↑GR ↑GST (cytosolic) ●GST (microsomal) ↑SOD ●TBARS	Griboff et al. (62)
Fly – *Drosophila melanogaster*, embryo-larval stage, wild type strains	experimental medium	The embryos were allowed to develop until emergence	0.01 mmol L^{-1} 0.1 mmol L^{-1}	↓ACAP (male) ↑ACAP (0.01 mmol L^{-1}-female) ↓ACAP (0.1 mmol L^{-1} -female) ●ROS (larvae, male) ↑ROS (0.01 mmol L^{-1}-female) ↑LPO (0.1 mmol L^{-1} -female)	Figueira et al. (63)
Honeybees	food	10 days	1.25 ng per bee 2.50 ng per bee 5.0 ng per bee	●TBARS	Helmer et al. (64)
Honeybees	food	1 day	0.1 µg L^{-1} 1 µg L^{-1} 10 µg L^{-1}	↓GPx (1,10 µg L^{-1}) ↓GSH ↓GST (1,10 µg L^{-1}) ↑MDA ↓TAA	Williams (65)
Male mice	intraperitoneally	14 days	78.25 mg kg^{-1}	↓CAT (liver, kidney) ↓GSH (liver) ↑GST (liver) ↓SOD (liver, kidney) ↑MDA (liver) ↓FRAP (liver)	EL-Shenawy et al. (66)
Male and female Balb/c mice	gavage	21 days	100 mg kg^{-1} 200 mg kg^{-1} 400 mg kg^{-1}	↓GSH ↑GSSG ↑ROS	Gao et al. (67)

Experimental model	Route of administration	Exposure duration	Concentration / dose	Oxidative stress markers	Ref.
Male Wistar rats	gavage	7, 14 and 21 days	300 mg kg^{-1}	↑CAT ↑GPx (after 14 and 21 days) ↓GSH ↑GST ↑SOD (after 14 and 21 days)	Singh et al. (68)
Adult male Wistar rats	gavage	16 days	120 mg kg^{-1} 200 mg kg^{-1}	↓CAT (epididymis) ●GSH (testes and epididymis) ↓GST (testes and epididymis) ↓SOD (testes and epididymis) ↑MDA (testes and epididymis)	Abarikwu et al. (69)
Male Wistar rats	gavage	7, 14 and 21 days	300 mg kg^{-1}	↑CAT (after 14 and 21 days) ↑GPx (after 14 and 21 days) ↓GSH ↑GST ↑SOD (after 14 and 21 days) ↑MDA	Singh et al. (70)
Peripubertal male Wistar rats	gavage	27 days	50 mg kg^{-1} 200 mg kg^{-1}	↓CAT (200 mg kg^{-1}- interstitial testicular cells) ↓GPx (200 mg kg^{-1}- interstitial testicular cells) ↑GPx (liver) ↑GST (200 mg kg^{-1} -liver) ↓GST (interstitial testicular cells) ●SOD ●TBARS	Pogrmić-Majkić et al. (71)
Male Wistar rats		14 days	300 mg kg^{-1}	↑CAT ↑GPx ↑GST ↓GSH ↑SOD ↑MDA	Shirisha et al. (72)
Adult Wistar rats	orally administered	16 days	120 mg kg^{-1}	↓CAT (brain, liver, kidney) ↑GSH (liver) ↑SOD (brain) ↓SOD (kidney, liver) ↑MDA (brain, liver, kidney)	Abarikwu (73)

Experimental model	Route of administration	Exposure duration	Concentration / dose	Oxidative stress markers	Ref.
Male Wistar rats	orally administered	15 days	0.3 mg kg^{-1}	●CAT (liver and kidney) ●GPx (liver and kidney) ●GSH (liver and kidney) ●SOD (liver and kidney) ●MDA (liver and kidney)	Jestadi et al. (74)
Male Wistar rats	orally administered	15 days	0.3 mg kg^{-1}	●CAT (testis) ●GPx (testis) ●GSH (testis) ●GST (testis) ●SOD (testis) ●MDA (testis)	Jestadi et al. (75)
Female Wistar rats	gavage	28 days	5 mg kg^{-1} 25 mg kg^{-1} 125 mg kg^{-1}	↓CAT (125 mg kg^{-1}) ↓GPx (5, 125 mg kg^{-1}) ●SOD ↑MDA (125 mg kg^{-1})	Liu et al. (76)
Male Wistar rats	gavage	3 times a week/52 days	12.5 mg kg^{-1}	●CAT (testis and liver) ●GPx (testis and liver) ●GSH (testis and liver) ●GST (testis and liver) ●SOD (testis and liver) ●MDA (testis and liver)	Abarikwu et al. (77)
Male Wistar rats	gavage	21 days	50 mg kg^{-1} 100 mg kg^{-1} 300 mg kg^{-1}	↓CAT (100, 300 mg kg^{-1} epididymis) ↑CAT (300 mg kg^{-1} testis) ↑GPx (epididymis) ↓GPx (100, 300 mg kg^{-1} testis) ↑GSH (300 mg kg^{-1} epididymis; 100, 300 mg kg^{-1} testis) ↓GST (300 mg kg^{-1} epididymis; 100, 300 mg kg^{-1} testis) ↑SOD (100, 300 mg kg^{-1} epididymis) ↓SOD (testis) ●MDA (epididymis, testis)	Abarikwu et al. (78)
Adult male albino rats	gavage	28 days	300 mg kg^{-1}	↓CAT ↓GPx ↓GSH ↓SOD ↑MDA	Abass et al. (79)
Male Wistar rats	NA	10 days	25 mg kg^{-1}	↓CAT ↓GSH ↓SOD ↑MDA	Adedosu et al. (80)

ACAP – antioxidant capacity against peroxide radicals; CAT – catalase; GPx – glutathione peroxidase; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidised glutathione; GST – glutathione S-transferase; LPO – lipid peroxidation levels; MDA – malondialdehyde; ROS – reactive oxygen species; SOD – superoxide dismutase; TAA – total antioxidant activities; TBARS – thiobarbituric acid reactive species

● – no change in oxidative stress marker; ↑ – increase; ↓ – decrease

GSH and the activities of CAT, SOD, GPx, and GST (74, 75, 77).

INVOLVEMENT OF OXIDATIVE STRESS IN TERBUTHYLAZINE TOXICITY

Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine) has become the key triazine in Europe in the last two decades (37). It is used on a variety of crops, such as cereals, legumes, and under fruit trees as a selective pre- and early post-emergence control agent against most grasses and many annual broadleaf weeds. It is also used as an aquatic herbicide to control submerged and free floating weeds and algae in water courses, reservoirs, and fish ponds (84).

According to the European Food Safety Agency (EFSA), it poses a high risk to non-target plants in the off-field areas, while the risk for bees, soil micro- and macro-organisms, and organisms used in biological wastewater treatment is low (85). EFSA has also warned about high toxicity of both terbuthylazine and its metabolite desethyl-terbuthylazine to aquatic organisms. In mammals exposed through the oral, dermal, or inhalation routes its acute toxicity can be low to moderate and cause a slight eye and skin irritation and sensitisation. Short-term exposure may affect body weight and food consumption in rats, mice, dogs, and rabbits. Long-term exposure may further affect organ weights in rats and mice and haematological parameters in rats (85).

According to the current cancer classifications, terbuthylazine belongs to Group D – "Not Classifiable as to Human Carcinogenicity" (86). There are several routes of human exposure to terbuthylazine. Occupational exposure occurs through inhalation and dermal contact at workplaces and commercial/industrial settings where terbuthylazine is produced or used. The general population is mostly exposed through ingestion of contaminated drinking water and dermal contact (87). Terbuthylazine has an extensive metabolic pathway. Its major degradation products (desethyl, hydroxy, and desethyl-hydroxy-terbuthylazine) have been identified in groundwater above the drinking water limit of 0.1 μg L^{-1} (85).

This herbicide persists in the environment and has a tendency to easily move from treated soils to water compartments through runoffs and leaching (88, 89). This has raised concern about its potential toxic effects on aquatic organisms. Table 2 summarises the findings about the affected markers of oxidative stress reported in recent studies on aquatic organisms. However, even though this is a relevant topic, considering that levels as low as 0.1 μg L^{-1} of terbuthylazine and its metabolites in freshwater pose a risk for non-targeted aquatic species, the number of such studies is scarce. Since 2010, only a few studies have been published on common carp, zebrafish, the red swamp crayfish (*Procambarus clarkii*), and marbled crayfish (*Procambarus fallax f. virginalis*) (45, 47, 90-97). Most investigated sub-chronic exposure to terbuthylazine at environmentally relevant concentrations and compared them with exposure to higher concentrations (70-1000 μg L^{-1}). A 28-day exposure of zebrafish to the environmentally relevant concentration of 0.55 μg L^{-1} of terbuthylazine did not affect oxidative stress biomarkers, while moderate to high concentrations (>400 μg L^{-1}) caused a significant decrease in TBARS and GR activity and increase in GST (93). The same was noticed in common carp exposed to 0.38 μg L^{-1} for 91 days, as terbuthylazine did not affect the GSH levels or GST activity (92).

Beside exposure to terbuthylazine or its commercial preparation Click 500 SC, aquatic experimental models were exposed to terbuthylazine degradation products, because they pose an even greater risk of water contamination due to high polarity (99). Stara et al. (98) reported higher levels of TBARS and changes in the activities of SOD, CAT, and GR in red swamp crayfish after a 14-day exposure to the environmentally relevant terbuthylazine-desethyl concentration of 2.9 μg L^{-1} (reported in Czech rivers) and the 200 times higher concentration (580 μg L^{-1}).

Several studies investigated the effects of terbuthylazine and its degradation products on developing fish (embryos and larvae) under laboratory conditions. Hostovsky et al. (90) reported higher GR activity only when the common carp embryos and larvae were exposed to the higher terbuthylazine concentration (520 μg L^{-1}) for 30 days. In contrast, Velisek et al. (94) reported lower SOD activity even from exposure to the lowest concentration of terbuthylazine-2-hydroxy (2.9 μg L^{-1}), whereas CAT and GR activities were not affected. Similar results for SOD were reported for a 31-day terbuthylazine-desethyl exposure starting with 1.8 μg L^{-1}, while no significant difference was found in TBARS, CAT, and GR (95). The same group of authors also investigated the effects of environmentally relevant concentrations of terbuthylazine 2-hydroxy (0.73 μg L^{-1}) and terbuthylazine-desethyl (1.80 μg L^{-1}) alone and in combination (97). After 36 days of exposure, terbuthylazine-desethyl lowered the SOD and GR activities in embryos and larvae of common carp, whereas terbuthylazine 2-hydroxy and their combination showed no effects. TBARS, CAT, GST, and GSH were not affected at all. In another Velisek et al. study (47), the same metabolites alone or in combination caused a significant increase in CAT activity developing marbled crayfish exposed for 77 days, whereas TBARS levels and SOD and GR activities remained in control range. High concentrations of terbuthylazine-2-hydroxy (375 and 750 μg L^{-1}) resulted in a significant decrease in TBARS levels and SOD activity after 62 days of exposure of marbled crayfish, but did not significantly affect CAT and GR activity (96).

Very little is known about effects of terbuthylazine on oxidative stress parameters and antioxidant defence in mammals. Recently, we reported that oral 28-day exposure to 0.004, 0.4, and 2.9 mg kg^{-1} bw day^{-1} of terbuthylazine disturbed the oxidant/antioxidant balance in Wistar rats (100). Namely,

total antioxidant capacity, expressed as plasma FRAP, significantly increased at 0.004 and 0.4 mg kg^{-1} bw day^{-1}. Plasma SOD dropped at 0.004 and 0.4 mg kg^{-1} bw day^{-1}, and plasma CAT at 2.9 mg kg^{-1} bw day^{-1}, whereas erythrocyte SOD increased at 2.9 mg kg^{-1} bw day^{-1}, and whole blood GPx at 0.4 mg kg^{-1} bw day^{-1}. Exposure to terbuthylazine did not induce significant lipid peroxidation.

EPIDEMIOLOGICAL STUDIES

Several studies in humans reported an imbalance between oxidative stress markers and the antioxidant defences in farmers/pesticide applicators or agricultural workers (7, 100-102). However, a thorough search of relevant literature yielded only one epidemiological study of the association between the biomarkers of atrazine exposure and biomarkers of oxidative stress (103), and none about terbuthylazine. In that one study, no association was found between atrazine mercapturate (AM) and MDA, 8-hydroxy-2'-deoxyguanosine (8-OHdG), or 8-isoprostaglandin-F2 (8-isoPGF) in urine samples collected from 30 farmers over five time points of atrazine application. However, when the authors excluded the samples with AM below the detection limit, they established a significant association between AM and 8-OHdG. These biomarkers did not vary significantly across the five time points. The authors noted that the measured biomarkers were immediate or short-term responses to environmental exposures, with a lag of up to a few days and that the measured metabolite reflected recent exposure, due to its short half-life.

Human exposure to atrazine or terbuthylazine results in a relatively fast urinary excretion of the parent compounds and their dealkylated and conjugated metabolites (104, 105). The half-life of atrazine, for example, is only 24-31 h (106, 107). The parent compound is, therefore, generally detected only in minor amounts in the urine, usually after continuous occupational exposure. In one study (108), for example, atrazine urine ranged between 5 to 29 µg L^{-1}after seven months of continuous work in atrazine production. In another study in corn farmers from Iowa, USA (109), the level of its metabolite AM in urine collected during crop planting of 1.0 µg g^{-1} of creatinine significantly correlated with the amount of atrazine applied per farmer over one year of 217 (47-530) kg. AM was also detected in only 23 % of non-farm family members from Iowa, USA, with geometric means of 0.067 µg L^{-1} in fathers, 0.031 µg L^{-1} in mothers, and 0.054 µg L^{-1} in children (110). Interestingly, however, Chevrier et al. (111) detected atrazine (0.12 µg L^{-1}, max. 0.52 µg L^{-1}) and AM (0.05 µg L^{-1}, max. 0.68 µg L^{-1}) in the urine of 5.5 % of 579 pregnant women in France for as long as three years after atrazine was banned in Europe.

Terbuthylazine, in turn, was detected in the hair of all farmers, with median and range of 0.61 (0.07-4.64) ng mg^{-1} of hair, but in only 67 % of rural residents, with median and range of 0.01 (<0.01-0.04) ng mg^{-1} of hair (112).

A number of studies evaluated health effects of atrazine exposure in humans. Most of them were retrospective and relied on the levels of atrazine in drinking water (38, 113-116), pesticide use records (117), or self-reported information (118, 119), which, of course, excluded determination of exposure on individual level. Moreover, these studies could not determine whether the observed outcomes were specific to atrazine or co-exposure to other pesticides or other compounds in the environment. Accurate assessment of human exposure is one of the major challenges to epidemiological studies of health effects from pesticide exposure (109). Exposure in farmers is characterised by large seasonal differences in the type, amount, and frequency of pesticide application, as well as application technique, formulations used, environmental conditions, and personal behaviour of pesticide applicator (120, 121). It is particularly challenging to asses exposure for individuals living in the vicinity of pesticide-treated fields or for the general population and to link it with health effects.

Some experimental studies have raised concern that atrazine can induce cancer in humans. However, epidemiological studies that investigated atrazine-related cancer risk have provided no evidence to support this causal relationship, at least judging by the studies looking for associations between atrazine exposure and non-Hodgkin lymphoma or prostate, ovarian, and breast cancer (1, 10, 122). The major limitation of these epidemiological studies is their inability to reliably assess past exposures. Furthermore, there is no biomarker so specific to reliably establish the link between a certain pesticide and a disease endpoint, which remains the challenge for future studies.

CONCLUSIONS

Triazines and their residues have been reported in various environmental media, including food and water. Furthermore, s-triazines have been identified as substances hazardous to the aquatic environment and have been included in the EU Priority Pollutants List (123). However, current knowledge about triazine toxicity is still inconclusive.

From the knowledge summarised in Table 1 we can conclude that atrazine generally does not affect lipid peroxidation in developing and adult aquatic organisms, but does set in motion the antioxidant defences (CAT, SOD, GPx, and GST) at low, environmentally relevant concentrations. These defences, however, sometimes cannot compensate for stress at high atrazine concentrations.

Literature reports on aquatic organisms summarised in Table 2 suggest that environmentally relevant concentrations of terbuthylazine do not generally affect the oxidant/antioxidant balance, but its metabolites do.

In other environmentally relevant organisms, such as drosophila and honeybees, atrazine turned out to be more of a concern because of evident lipid peroxidation and antioxidant depletion.

Table 2 *Changes in oxidative stress markers in experimental models after exposure to terbuthylazine and its metabolites terbuthylazine-2-hydroxy and terbuthylazine-desethyl*

Compound / concentration	Experimental model	Exposure duration	Oxidative stress markers	Ref.
Terbuthylazine				
0.9 μg L^{-1} 160 μg L^{-1} 520 μg L^{-1} 820 μg L^{-1}	Fish – common carp, embryos and larvae	30 days	↑GR (520 μg L^{-1}) ●GST ●TBARS	Hostovsky et al. (90)
0.38 μg L^{-1} 60 μg L^{-1} 550 μg L^{-1}	Fish – common carp	91 days	●GSH ●GST ↑FRAP (550 μg L^{-1})	Mikulikova et al. (91)
3300 μg L^{-1}	Fish – common carp	24 hours	●FRAP	Mikulikova et al. (92)
0.55 μg L^{-1} 150 μg L^{-1} 400 μg L^{-1} 700 μg L^{-1} 1000 μg L^{-1}	Fish – zebrafish	28 days	●GPx ↓GR (700, 1000 μg L^{-1}) ↑GST (400, 700, 1000 μg L^{-1}) ↓TBARS (1000 μg L^{-1})	Plhalova et al. (93)
Terbuthylazine-2-hydroxy				
2.9 μg L^{-1} 70 μg L^{-1} 1400 μg L^{-1} 3500 μg L^{-1}	Fish – common carp, embryos and larvae	35 days	●CAT ●GR ↓SOD	Velisek et al. (94)
0.73 μg L^{-1}	Fish – common carp, embryos and larvae	36 days	●CAT ●GSH ●GST ●TBARS	Velisek et al. (95)
0.73 μg L^{-1}	Marbled crayfish eggs	77 days	●CAT ●GR ●SOD ●TBARS	Velisek et al. (47)
0.75 μg L^{-1} 75 μg L^{-1} 375 μg L^{-1} 750 μg L^{-1}	Marbled crayfish eggs	62 days	↓SOD (375, 750 μg L^{-1}) ●CAT ●GR ↓TBARS (375, 750 μg L^{-1})	Koutnik et al. (96)
Terbuthylazine-desethyl				
1.80 μg L^{-1} 180 μg L^{-1} 900 μg L^{-1} 1800 μg L^{-1}	Fish – common carp, embryos and larvae	31 days	●CAT ●GR ↓SOD ●TBARS	Velisek et al. (97)
1.80 μg L^{-1}	Fish – common carp, embryos and larvae	36 days	●CAT ↓GR ●GSH ●GST ↓SOD ●TBARS	Velisek et al. (95)
1.80 μg L^{-1}	Marbled crayfish eggs	77 days	↑CAT ●GR ●SOD ●TBARS	Velisek et al. (47)

2.9 μg L^{-1} 580 μg L^{-1}	Adult red swamp crayfish	14 days	↑CAT (muscle) ↑GR (580 μg L^{-1} muscle) ↑GSH (580 μg L^{-1}hepatopancreas) ↑GST (hepatopancreas) ↓SOD (muscle) ↑SOD (580 μg L^{-1} hepatopancreas) ↑TBARS (hepatopancreas)	Stara et al. (98)

CAT – catalase; FRAP – ferric reducing ability of plasma; GPx – glutathione peroxidase; GR – glutathione reductase; GSH – reduced glutathione; GST – glutathione S-transferase; SOD – superoxide dismutase; TBARS – thiobarbituric acid reactive species
● – no change in oxidative stress marker; ↑ – increase; ↓ – decrease

As for rodents, laboratory studies have confirmed the associations between the two triazines and oxidative stress found in aquatic organisms. They generally suggest that the antioxidant enzyme defences in rodents are compromised.

Although individual biomarkers can be useful as early warning signals, a combination of several markers is more useful to understand the mode of action of a pesticide and identify possible molecular targets. Supporting these notions, the reviewed studies have demonstrated that there is no single specific biomarker of oxidative stress caused by atrazine or terbuthylazine and that a complex approach should be taken to assess oxidative response to pesticides.

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Conflicts of interest

None to declare.

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Oksidacijski stres u toksičnosti triazinskih pesticida - pregled glavnih biomarkera

Ovaj pregledni članak daje sažetak studija objavljenih od 2010. godine o djelovanju atrazina i terbutilazina na parametre oksidacijskog stresa (osobito lipidnu peroksidaciju i antioksidacijske enzime) u eksperimentalnih životinja i ljudi. Općenito je utvrđeno da izloženost eksperimentalnih životinja atrazinu i terbutilazinu najviše utječe na njihovu antioksidacijsku obranu i u manjoj mjeri na lipidnu peroksidaciju, ali učinci variraju ovisno o vrsti, spolu, dobi, koncentraciji herbicida i trajanju izlaganja. Većina studija uključivala je vodene organizme kao korisne i osjetljive bio-pokazatelje onečišćenja okoliša i važan dio prehrambenog lanca. U laboratorijskih miševa i štakora promjene u markerima oksidacijskog stresa bile su vidljive samo nakon izloženosti visokim dozama atrazina. U svojem smo nedavnom izvješću iznijeli podatak da niske doze terbutilazina također mogu inducirati oksidacijski stres u Wistar štakora. Očigledno je da pri svakoj eksperimentalnoj procjeni toksičnih učinaka pesticida treba uzeti u obzir kombinaciju nekoliko biomarkera oksidacijskog stresa i antioksidacijske obrane u različitim tkivima i stanicama. Učinci identificirani u eksperimentalnim modelima trebaju se nadopunjavati i potvrditi epidemiološkim istraživanjima. To je važno ako želimo razumjeti utjecaj pesticida na ljudsko zdravlje i uspostaviti sigurnu granicu izloženosti.

KLJUČNE RIJEČI: *atrazin; antioksidacijski enzimi; lipidna peroksidacija; terbutilazin*