# Effects of the exposure of fish to triazine herbicides

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## Abstract

Triazines are an important group of herbicides, which have been used extensively or selectively both in agricultural and non-agricultural areas worldwide for more than fifty years to control broadleaf and some grassy weeds. As a consequence of their massive application, they have become and remain significant environmental pollutants, especially in aquatic ecosystems. Fish are an integral part of the aquatic environment and are, therefore, suitable models for the study of the behavioral, biological, and biochemical effects of triazine exposure. We have summarized and evaluated the effects of triazine herbicides on fish in order to provide an overview of current information on triazines. The overall effects of triazine herbicide exposure on the physiology of fish were evaluated by considering a variety of parameters in a number of reports. Haematological and biochemical profiles of blood provide important information about the internal environment of the organism and the general physiology and health status of triazine exposed fish. According to studies using biotransformation and bioaccumulation indices to estimate the effects of triazines on fish, changes in fish metabolism reflect the pollution of the environment by these herbicides. The responses of antioxidant defence systems in fish to triazine exposure could be an adaptive mechanism to protect the fish from triazine-induced oxidative stress. Acute exposure to triazines affects reproduction or reproductive development in fish; however, some triazines did not affect fish behavior during long-term exposure to low concentrations. Nevertheless, an impact on the overall behavioral response of fish cannot be excluded.

**Abbreviations:**

| | |
|---|---|
| ALT | - alanine aminotransferase |
| AST | - aspartate aminotransferase |
| CAT | - catalase |
| CHOL | - cholesterol |
| CYP | - cytochrome |
| EPA | - Environmental Protection Agency |
| EROD | - ethoxyresorufin-O-deethylase |
| EUREAU | - European Union of National Associations of Water Suppliers and Waste Water Services |
| GLU | - glucose |
| GPx | - glutathione peroxidase |
| GR | - glutathione reductase |
| GSH | - glutathione |
| GSSG | - glutathione disulfide |
| GST | - glutathione S-transferase |
| Hb | - haemoglobin concentration |
| LC50 | - lethal concentration 50% |
| Leuko | - leukocyte count |
| Leukogram | - differential leukocyte count |



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LOEC - Lowest observed effect concentration
LOOH - fatty acid peroxides
LPOX - lipid peroxidation
MATC - maximum acceptable toxicant concentration
MCH - mean corpuscular haemoglobin
MCHC - mean corpuscular haemoglobin concentration
MCL - maximum contaminant level
MCV - mean corpuscular volume
MDA - malondialdehyde
NADPH - nicotinamide adenine dinucleotide phosphate (reduced form)
NOEC - No observed effect concentration
PCV - packed cell volume, haematocrit
RBC - erythrocyte count
SOD - superoxide dismutase
TBARS - thiobarbituric acid reactive substances
TP - total protein
WBC - white blood cells count

## INTRODUCTION

Environmental pollution by pesticides has become a serious problem in terms of global conservation and animal and human health (Katsumata *et al.* 2005; Velisek *et al.* 2010). Pesticides are ubiquitous agricultural chemicals and can spread regionally and globally into many ecosystems. Over the last few decades the uses of pesticides have dramatically increased in relation to increasingly intensive agricultural practices. As a consequence of this massive use of pesticides in agriculture, they have become a significant ecological burden especially in aquatic ecosystems (Cerejeira *et al.* 2003; Flynn & Spellman 2009).

An important group of herbicides are triazines, which have been used extensively or selectively primarily to control broadleaf and some grassy weeds in agriculture and in non-agricultural areas worldwide for more than 50 years (Moretti *et al.* 2002; Arufe *et al.* 2004; Breckenridge *et al.* 2008). The application of pesticides such as herbicides or insecticides, and their mixtures, may often result in negative impacts on non-target organisms in the aquatic environment, including fish (Di Giulio & Hinton 2008; Slaninová *et al.* 2009; Zhang *et al.* 2010).

For many reasons, fish species are very important organisms in studies assessing biological or biochemical responses to environmental contaminants including pesticides (Powers 1989; Ostrander 2000; Van der Oost *et al.* 2003). Fish play an important role in the ecosystem and can be found virtually everywhere in the aquatic environment, in which they are an important link in the food chain, and, therefore, generally considered to be the most viable organisms for monitoring pollution in the water environment. Understanding the absorption, behavior and reactions of pesticides in the fish organism has, therefore, high ecological relevance (Van der Oost *et al.* 2003; Slaninová *et al.* 2009).

This review contains individual chapters, each with a specific focus on triazine herbicide characteristics or their impact on fish. Overall conclusions evaluate important data about toxicity and the physiological and morphological alterations that arise from triazine exposure in the laboratory or in the field. References and sources are considered on the basis of their importance in relation to fish toxicology and ecotoxicology generally. The aim of our work was to assess the potential risks of triazine herbicide exposure to fish in accordance with the evidence.

## CLASSIFICATION AND CHEMISTRY OF TRIAZINE PESTICIDES

Triazine compounds are divided into two main groups according to their chemical structure: symmetrical triazines (1,3,5-triazines) and asymmetrical triazinones (1,2,4-triazionones; e.g. metribuzin, metamitron, hexazinone, amibuzin). The symmetrical triazines (s-triazines) are major commercial herbicides and can be further divided into chloro-s-triazines (e.g. atrazine, simazine, terbuthylazine, propazine, cyanazine, sebuthylazine), thiomethyl-s-triazines (e.g. ametryn, prometryn, terbutryn, simetryn, desmetryn), fluoroalkyl-s-triazines (e.g. indaziflam and triaziflam), and andmethoxy-s-triazines (e.g. atraton, prometon, simeton, terbumeton). Other herbicides with a triazin-like structure are triazoles (e.g. amitrole and epronaz) and triazolones (e.g. amicarbazone and bencarbazone) (Roberts *et al.* 1998; Stevens *et al.* 2001; Appleby *et al.* 2005; Breckenridge *et al.* 2010).

The structure of triazines comprises a heterocyclic ring, similar to the six-membered benzene ring but with nitrogen atoms replacing three carbons. Most of the symmetrical triazines have a sulphur, chlorine, or oxygen atom at the 2-position of the ring and are usually substituted with alkylamino-groups in the 4- and 6-positions. The cyanazine molecule contains a 2-cyano-isopropylamino-substituent at the 4-position on the ring (Stevens *et al.* 2001; Appleby *et al.* 2005; LeBaron *et al.* 2008).

The procedure for the production of triazines is the reaction of cyanuricchloride with ethylamine, followed by treatment with isopropylamine (e.g. atrazine, simazine, cyanazine, terbuthylazine). Some triazine agents are produced by the reaction of other triazines with methylmercaptan in the presence of sodium hydroxide (e.g.prometryn, terbutryn, ametryn) (Appleby *et al.* 2005).

## ABSORPTION, MODE OF ACTION IN PLANTS AND USE OF TRIAZINE HERBICIDES

The absorption of triazine herbicide compounds by plants is mediated primarily by the roots but also by the leaves, and they are transported upwards in the xylem and accumulated in the apical meristems. Thereafter, they move into the green leaf tissue, where they cause the leaves to turn yellow and die. Because of the low

solubility of triazine substances in water, they usually do not penetrate down to the deeper levels of soils and thus have little effect on deep-rooted plants, which is one of the factors governing their selective application (Roberts *et al.* 1998; Waxman 1998; Appleby *et al.* 2005; Simoneaux & Gould 2008).

The metabolic processes of triazines in plants seem to be very complex exhibiting a great variety of biological reactions. The main three metabolism pathways in plants are 1) non-enzymatic hydrolytic dehalogenation at the chloro- or 2-position of the triazine ring; 2) conjugation with glutathione, forming s-bound amino acid conjugates; and 3) side-chain de-alkylation (at the 4- or 6-position of the triazine ring) or 4) side chain oxidation to 2-hydroxy derivates (Roberts *et al.* 1998; Simoneaux & Gould 2008).

Triazine herbicides belong to the group of photosynthesis inhibitors designed for organisms with oxygen evolving photosystems. The primary mode of action is the inhibition of the Hill reaction phase of photosynthesis electron transport by the displacement of plastoquinone from a specific-binding niche on the D1 protein subunit of photosystem II (see Fig.1). If the triazine exposure level is below the threshold of photosynthesis inhibition, this action is fully reversible (Waxman 1998; Das *et al.* 2000; Appleby *et al.* 2005; Trebst 2008).

Various chemicals have been used since ancient times to control unwanted vegetation and weeds. There are many benefits to using chemicals for weed control compared to physical or biological methods and, therefore, herbicides such as triazines are used extensively worldwide. The herbicidal activity of triazines was discovered by J. R. Geigy Limited (a Swiss company) and they developed the first triazine pesticide, simazine (Kamrin 1997), which was approved in Switzerland in 1956. Thus, triazines are some of the oldest synthetic herbicides and have a wide range of uses (Waxman 1998; Appleby *et al.* 2005; Velisek *et al.* 2008). The introduction of triazine herbicides amounted to a revolution in weed control with respect to corn and grain sorghum. The first s-triazines, simazine and atrazine, were applied mostly to maize, but the use of these two herbicides, as well as other triazine variants, has expanded to selective applications in perennial crops, fruit, and non-crop agriculture. Even today, atrazine remains the leading corn herbicide (Tomlin 1994; Appleby *et al.* 2005; LeBaron *et al.* 2008). Triazine herbicides are regarded to be the most commonly used herbicides in the USA, Europe and in many places around the world, mostly in the treatment of maize crops (Grube *et al.* 2011; FAO 2013).

Triazine compounds are primarily used as selective, systemic herbicides, pre- and post-emergence, to control annual broad-leaved weeds and annual grasses in a general broad spectrum of agricultural crops and also to control industrial weeds. For example, atrazine is used in maize on account of its much more rapid degradation in the crop than in weeds; prometryn is used in cotton and vegetables; propazine is applied in sorghum or umbelliferous crops. Other triazine herbicides, such as simazine, cyanazine and terbutryn, are used as broad spectrum herbicides in the culture of a variety of crops, legumes, coffee, potatoes, vines, and tree fruits; terbuthylazine is applied in forestry. The herbicidal activity of metribuzin, an asymmetrical triazinone, is selectively used in a wide range of corns, legumes (mainly soybean), and vegetables, and in forestry chemisation (Pauli *et al.* 1990; Roberts *et al.* 1998; Fairchild & Sappington 2002; Tomlin 1994; Appleby *et al.* 2005).

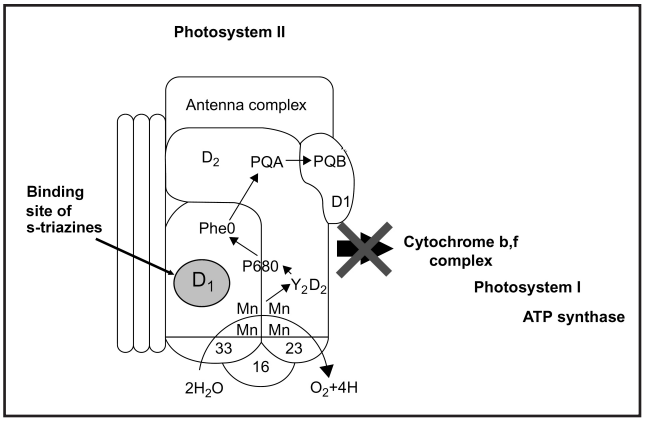


**Fig. 1.** Specific-binding site of triazines on the D1 protein subunit of photosystem II (Swindell 2003).

In addition to agriculture, triazine compounds were and some of them still are also used in aquaculture to control unwanted weeds, as well as algicides in ponds, water reservoirs and swimming pools. Terbutryn was commonly used as a herbicide or algicide in the control of submerged and free-floating weeds and algae in aquaculture farming (Nilson & Unz 1977; Tomlin 1994), as well as an organic biocide in the organic facade of coating systems (Burkhardt *et al.* 2012). Simazine is also used as an algicide in fish farm ponds, aquariums, and cooling systems (Jones & Burch 1997; Gunasekara *et al.* 2007) and as a constituent (10–30%) of an internationally patented algicide for open circulatory cooling water systems (Chen *et al.* 2009).

## TRIAZINES IN THE AQUATIC ENVIRONMENT

Whether alone or in combination with other herbicide compounds, triazines are still among the largest selling and most widely used herbicides worldwide. Simazine is one of the oldest synthetic pesticides and for more than five decades symmetrical triazines (s-triazines) have been used as selective herbicides to control weeds (Appleby *et al.* 2005; Breckenridge *et al.* 2008). Herbicides such as atrazine, simazine, and cyanazine are considered to be the most important and most commonly used triazine-based herbicides in the USA and in many places all over the world (Grube *et al.* 2011; FAO 2013).

These pesticides are applied in thousands of tons per acre of farmland worldwide, which has resulted in the contamination of surface and ground water by triazines and their metabolites. Their levels in water compartments vary with the seasons according to the times when they are applied to crops. Because of their extensive application and appreciable persistence in soil, triazine herbicides have been confirmed as particularly troublesome in drinking water sources and, of course, also in food products (Muir & Baker 1976; Muir 1980; Konstantinou *et al.* 2006; Granby *et al.* 2008; Tillitt *et al.* 2010).

There are many interacting factors which influence the activity, movement, and persistence of triazine herbicides, including the texture and physical parameters of soil, climate, and, of course, chemical or biological degradation. The triazine ring is comparatively resistant to microbial or fungal breakdown and, therefore, biodegradation has a low environmental impact. Although photocatalytic and generally effective chemical degradation is described, these substances or their partial metabolites persist for one or more years in soil or sediments, according to the climatic conditions and level of application (Pelizzetti *et al.* 1990; Manahan 2005; Appleby *et al.* 2005; Solomon *et al.* 2008).

The movement, persistence, and degradation of triazines in soil have been thoroughly studied and well described. Their persistence is generally considered in terms of the time taken for 50% of the triazine to either degrade or dissipate from the soil matrix (half-life). Depending on many factors, triazines and their metabolites can remain in soil after applications for many days or even years (LeBaron 1970; Graymore *et al.* 2001; Koskinen & Banks 2008).

After atrazine treatment at an initial concentration of 100 μg $g^{-1}$ of soil, a half-life of up to 1162 days was reported in Australian soil (Bowmer 1991). According to one study, simazine slowly degraded with an anaerobic soil half-life of 91 days (Wackett *et al.* 2002). The half-life of metribuzin in soil with high organic matter content was reported to be up to 120 days (Roberts *et al.* 1998).

The excessive use of pesticides for weed control in field crops has also affected the aquatic environment. Significant amounts of triazine herbicides have been lost into water compartments through runoff from treated agricultural land. Their tendency to move and leach has been demonstrated, and residual amounts of triazines and their metabolites have been found in groundwater, seas, estuaries, rivers, and other surface waters or even groundwater in many regions around the world (US EPA 1994; Steinberg *et al.* 1995; Solomon *et al.* 1996; Oropesa *et al.* 2008; Caquet *et al.* 2013; Nödler *et al.* 2013). The environmental concentrations of triazine herbicides in the aquatic environment have been well reported over many years. It can be stated that the highest reported concentrations are often associated with rainfalls after application (Thurman *et al.* 1991, 1992; Thurman & Cromwell 2000; Du Preez *et al.* 2005; Yang *et al.* 2013; Otto *et al.* 2012).

Eight s-triazines (atrazine, simazine, sebuthylazine, prometryn, propazine, terbutryn, cyanazine and terbuthylazine) or their breakdown products have been identified as relevant hazardous substances, on the basis of the compilation of monitoring data from freshwaters in 15 member states of the European Union of National Associations of Water Suppliers and Waste Water Services (EUREAU) (EC 1999). An overview of the occurrence of selected triazine componds in the aquatic environment is presented in the following part.

*Atrazine*

Although the application of atrazine has been banned in many countries (in the EU region by European Commission Directive No. 2004/248/EC), because of its potential to bioaccumulate in organisms and its proposed negative effects, it is still one of the most widely used agricultural pesticides in the world applied before and after planting to control broadleaf and grassy weeds. Some governments have tried to reduce atrazine usage by enacting various laws, rules or guidelines, such as the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME 1999) or by establishing a Maximum Contaminant Level (MCL) for atrazine (3 μg $L^{-1}$) in drinking water (US EPA 2006), but atrazine and its degradation products (hydroxyatrazine, desethylatrazine, deisopropylatrazine, didealkylatrazine and deethylhydroxyatrazine) are still detectable in surface and ground waters (Thurman *et al.* 1991, 1992; Muller *et al.*1997; Panshin *et al.* 2000; Ren & Jiang 2002; Geng *et al.* 2013).

The presence of atrazine in water has long been studied, and, generally, residual levels of these pesticides are detected at the low ng $L^{-1}$ level. However, some reported concentrations exceed values of μg $L^{-1}$ because of its applications at rates of up to 20 kg/ha in areas of extensive agricultural activity. Reported values for atrazine concentrations in waters vary greatly with detectable concentrations ranging from 0.001 μg $L^{-1}$ up to values exceeding 100 μg $L^{-1}$ in the US "corn belt" and Canada; these reports generally involve surface runoff from agricultural land (Kucklick & Bidleman 1994; Graymore *et al.* 2001; Salvadó *et al.* 2006; Benotti *et al.* 2009). Atrazine concentrations of between 3 and 275 μg $L^{-1}$ were found in surface run-off water from agricultural land in USA or England (Southwick *et al.* 1990; Huber 1993; Environment Agency 1997). Reported levels in South Texas coastal waters reached 65 μg $L^{-1}$ (Thurman *et al.* 1992, Pennington *et al.* 2001). In China, atrazine concentrations exceeding the maximum contaminant level for atrazine in surface water (3 μg $L^{-1}$) were reported in the Guanting Reservoir (0.67–3.9 μg $L^{-1}$) (Ren *et al.* 2002) and Taihu Lake (7.12±0.54 μg $L^{-1}$) (Dong *et al.* 2006). According to the Czech Hydrometeorological Institute, high levels of atrazine residues are still found in Czech rivers; water samples exhibited maximum concentrations ranging from 0.3 to 1.0 μg $L^{-1}$ in the years

from 2005 to 2008 (CHMI 2011). Numerous studies indicate that atrazine continues to be a contaminant of surface and ground water in many countries through many years (Shipitalo *et al.* 1997; Miller *et al.* 1999; Van Maanen *et al.* 2001; Murphy *et al.* 2006; Gonçalves *et al.* 2007; Hildebrandt *et al.* 2008; Palma *et al.* 2009; Loos *et al.* 2010; Loos *et al.* 2013).

*Simazine*

According to The European Commission, Directive 196/2010/EC , simazine as an active ingredient of herbicides is banned for plant protection products in the countries of the European Union (OJ 2010); however, similarly to atrazine, simazine-based herbicides are still widely used in the USA (Battaglin *et al.* 2001). Simazine was reported as the most frequently detected pesticide in Californian waters with concentrations ranging from 0.02 to 49.2 $\mu g\,L^{-1}$ (US EPA 1994). Simazine was found in marine water samples (Adriatic Sea) taken 16 km offshore from Venice (Italy), and detected concentrations were higher during summer (up to 0.02 $\mu g\,L^{-1}$) (Loos *et al.* 2013). The concentrations of simazine in rivers and streams of the midwestern United States after the application of herbicides were studied. From 1989 to 1990, maximum values in the aquatic environment increased up to 8.7 $\mu g\,L^{-1}$ in the preplanting period and up to 7.0 $\mu g\,L^{-1}$ in the post-planting season. Thus, simazine levels were frequently 3–10 times higher than the established US EPA MCL (U.S. Environmental Protection Agency Maximum Contaminant Level for simazine – 0.001 $\mu g\,L^{-1}$) (Thurman *et al.* 1992). A similar rising trend after agricultural herbicide treatment was observed in the Llobregat River (Spain) in 2002; from a relatively low environmental concentration of 0.008 $\mu g\,L^{-1}$ in February, the level grew up to 2.218 $\mu g\,L^{-1}$ in April in the same river (Rodriguez-Mozaz *et al.* 2004). Pesticide concentrations were detected in river water at eight sampling sites in the Imathia agricultural region of northern Greece. The samples collected between May 1996 and April 1997 contained simazine at a maximum concentration of 0.317$\mu g\ L^{-1}$. The major inputs of triazine herbicides occurred in May and June just after the treatment of weeds (Albanis *et al.* 1998). Water samples from rivers running through agricultural areas in southern Spain contained simazine residue concentrations of up to 1.98 $\mu g\,L^{-1}$ (Belmonte *et al.* 2005).

A large study spanning 26 European countries was conducted in 2008; ground water samples were collected and analyzed and the highest simazine concentrations were found to be around 0.127$\mu g\,L^{-1}$ (Loos *et al.* 2010). Although the highest simazine concentration so far reported in surface water in the Czech Republic was 60 $\mu g\,L^{-1}$ (Velisek *et al.* 2009b), in a number of other European countries simazine concentrations of up to 4.5 $\mu g\,L^{-1}$ are regularly reported in surface and ground waters (Papadopoulou-Mourkidou *et al.* 2004; Oropesa *et al.* 2008; García-Galán *et al.* 2010).

*Sebuthylazine*

Environmental trace analysis of sebuthylazine in samples of water and soil from agricultural areas fertilized with sludge from wastewater treatment was used to evaluate the amount of triazines entering the aquatic environment from soil. Even though sebuthylazine was present in soil at high concentrations of up to 0.02 $\mu g\,kg^{-1}$, its concentrations in ground water samples were under the method's limit of detection (García-Galán *et al.* 2010). In a work investigating the presence of organic compounds in samples of wastewater effluent from the Mediterranean and Atlantic basins of Andalusia (Spain), triazine pesticides were not detected above 0.2 $\mu g\,L^{-1}$. Meanwhile, sebuthylazine was among the most commonly detected in polar pesticides class (Barco-Bonilla *et al.* 2013a, 2013b).

*Prometryn*

Although prometryn has been banned in many countries, similarly to other triazines, prometryn and its metabolites are still detected in aquatic ecosystems (Goolsby *et al.* 1993; Gfrerer *et al.* 2002; Silva *et al.* 2011; Qi *et al.* 2014). Triazine herbicides were routinely detected in samples from the Playa Lake receiving runoff from cotton-growing areas of the West Texas High Plains, and prometryn was one of the compounds exhibiting a relatively higher mean concentration of about 1.3$\mu g\ L^{-1}$ in water during the summer of 1997 (Thurman & Cromwell 2000). In sampling performed between 1992 and 2007, prometryn was detected in surface water of the Everglades agricultural area, exhibiting a maximum concentration of 0.59 $\mu g\,L^{-1}$ (Pfeuffer 2009). Prometryn was the most frequently detected pesticide found in the riparian drainage canal of the River Erythropotamos (Greece), with the highest median concentration of 0.19 $\mu g\,L^{-1}$ (Vryzas *et al.* 2011).

*Propazine*

Ground water samples collected in European countries contained propazine at a maximum detected concentration of 0.025 $\mu g\,L^{-1}$ (Loos *et al.* 2010). Between 2004 and 2006 propazine was shown to be one of the pesticides with the lowest frequency of detection in surface waters along the Ebro River, with a concentration of 0.182 $\mu g\,L^{-1}$ (Navarro *et al.* 2010). In spite of intensive pesticide use in the agricultural area of the Erythropotamos River (Greece), the median concentration of propazine found in the riparian drainage canal of the river was only 0.014 $\mu g\,L^{-1}$ (Vryzas *et al.* 2011). Maximum propazine concentrations in Spanish groundwater ranged between 0.01 and 0.1 $\mu g\,L^{-1}$ (Silva *et al.* 2006; Jurado *et al.* 2012). There are some reports by the U.S. Geological Survey of the detection of propazine and its metabolites in surface water and groundwater samples. Most of these findings were only obtained after storm flow and the concentrations were all below 0.5 $\mu g\,L^{-1}$ (Phillips *et al.* 1999, 2000).

*Terbutryn*

The use of terbutryn as a compound in plant protection products has been banned in many countries (for instance, by European Council Directive 91/414/EC and European Commission Regulation 2076/2002) also because of its potential to bioaccumulate in organisms (Rioboo *et al.* 2007). Nevertheless, due to its slow degradation, with a half-life of 180 and 240 days in pond and river sediments, respectively (Muir 1980), residual amounts of terbutryn and its metabolites have been found in aquatic environment long after application (Villarini *et al.* 2000; Moretti *et al.* 2002; Konstantinou *et al.* 2006; Baran *et al.* 2007; Nordmark *et al.* 2008; Velisek *et al.* 2010). Terbutryn levels in European waters range from 0.02 μg $L^{-1}$ to values reaching 5 μg $L^{-1}$ (Quednow & Püttmann 2007; Ormad *et al.* 2008; Velisek *et al.* 2010). Terbutryn at a concentration of 0.002 μg $L^{-1}$ was detected in Lake Geneva (Buser 1990); and, later, nearly the same value (0.003 μg $L^{-1}$) was found there between 2004 and 2005 (Gregorio *et al.* 2012). Triazine herbicides also represent a risk to marine aquatic environments, as terbutryn was also detected in Mediterranean coastal waters at a concentration up to 0.184 μg $L^{-1}$ (Tolosa *et al.*1996). The mean concentration reported for terbutryn in the Elbe River was 0.14 μg $L^{-1}$ for the period from 1982 until 1992; therefore, the annual riverine input via the Elbe in to the German Bight was estimated to be 78 tons per year (Huhnerfuss *et al.* 1997).

*Cyanazine*

There is little information in the literature on the aquatic occurrence and persistence of cyanazine, a fact that makes it difficult to evaluate its final impact on the aquatic ecosystem (Pacepavicius *et al.* 1996). Cyanazine was infrequently detected in groundwater samples collected in Iowa in 1999, with a concentration of about 0.02 μg $L^{-1}$. On the other hand, several cyanazine degradates were much more frequently detected in groundwater than their parent compound, with a maximum level of 3.3 μg $L^{-1}$ (Kolpin *et al.* 2001). Degradates of cyanazine also appeared more frequently than their parent compound in surface water and groundwater samples in other U.S. studies (Kolpin *et al.* 1998; Boxall *et al.* 2004). In the summary of concentrations of selected pesticides and pesticide degradation products determined in U.S. surface water samples collected during 2003–2004, cyanazine has a maximum concentration of 0.03 μg $L^{-1}$ and a mean concentration under <0.001 μg $L^{-1}$ (Scribner *et al.* 2006).

*Terbuthylazine*

Terbuthylazine concentrations in surface and ground waters ranged from 0.01 to 4.50 μg $L^{-1}$, with greater pesticide concentrations detected mainly during the seasons of application and after agricultural use (Brambilla 1993; Bossi *et al.* 2002; Lambropoulou *et al.* 2002; Belmonte *et al.* 2005; Hildebrandt *et al.* 2008; Fait *et al.* 2010; Chary *et al.* 2012; Jurado *et al.* 2012). In a large-scale European study of pollutants in EU ground waters, terbuthylazine was found at a concentration of 0.716 μg $L^{-1}$ (Loos *et al.* 2010). The highest reported environmental concentration of terbuthylazine in Czech rivers was 2.6 μg $L^{-1}$ (Kodes *et al.* 2010). The average yearly leachate concentrations of terbuthylazine and its degradation products in drainage water after the application of terbuthylazine exceeded 0.1 μg $L^{-1}$, and the presence of both terbuthylazine and desethyl-terbuthylazine were found in several drainage water samples many years after application in Denmark between 1999 and 2009 (Rosenbom *et al.* 2010). Terbuthylazine concentrations in German rainwater in 1992 were reported to range from 0.003 to 0.52 μg $L^{-1}$ (Hüskes & Levsen 1997).

*Metribuzin*

Metribuzin, like other triazine and triazinone herbicides, enters the aquatic environment via runoff and the contamination of water by metribuzin and its metabolites has been investigated by many authors. Environmental concentrations of metribuzin in waters are usually reported to be low, with maximum values under 0.1 μg $L^{-1}$ (Laabs *et al.* 2002; Kjær *et al.* 2005; Dores *et al.* 2006; Maloschik *et al.* 2007; Knee *et al.* 2010; Flores-García *et al.* 2011; Xing *et al.* 2012c); however, samples taken in the United States in 1998 exhibited higher metribuzin concentrations (1.76μg $L^{-1}$) (Battaglin *et al.* 2001). Metribuzin was the most frequently detected triazine compound in a study of the occurrence of herbicides in water samples collected in the Primavera do Leste region (Middle West of Brazil) during December 1998; maximum concentrations were 0.138 μg $L^{-1}$ in the reservoir and 0.351 μg $L^{-1}$ in a drinking water well (Dores *et al.* 2006). A modelling study indicated that metribuzin can reach concentrations of up to 390 μg $L^{-1}$ in surface water runoff (Pauli *et al.* 1990).

## TRIAZINE TOXICITY IN FISH

A large amount of toxicity data concerning triazine compounds is available from various sources, as might be expected for herbicides that are designed or use in terrestrial ecosystems, as well as in aquatic environments, and which have a tendency to move from treated soils into water compartments through runoff and by means of leaching. Toxicity studies of the effects of triazine herbicides on fish have revealed that they cause many morphological, physiological and biochemical changes (Saglio & Trijasse 1998; Arufe *et al.* 2004; Dezfuli *et al.* 2006; Nieves-Puigdoller *et al.* 2007; Mikulikova *et al.* 2011).

The toxicity of triazine substances to fish depends on various factors, such as species, age, body weight and condition, intermediary metabolism rate, and the chemical, biological and biophysical parameters of the water (Johnson & Finley 1980; Velisek *et al.* 2008; Plhalová *et al.* 2009, 2010). The nature of individual formulations and their chemical structures may affect the solubilisa-

tion and final toxic effects of triazines to an unknown degree, and, therefore, the mentioned endpoints of lethal or effective concentrations may vary considerably (Turner 2003; Appleby *et al.* 2005; Solomon *et al.* 2008). Because triazine compounds have their primary effect on unique plant systems, toxicity to vertebrates is generally considered to be very low (CCME 1999; Appleby *et al.* 2005). Assessment of the ecotoxicological risks of such pesticides is based on toxicity data and the effects of triazine compounds on fish organisms such as acute effects or profound chronic effects on development, growth, and morphology (Wiegand *et al.* 2000; Velisek *et al.* 2009a, 2009b; Plhalová *et al.* 2010).

*Acute toxicity*

There are numerous studies on the acute toxic effects of triazines on fish. The results evaluate triazines as falling under the practically non-toxic and moderately toxic categories according to qualitative descriptors for categories of fish. Acute fish toxicity data are presented in Table 1.

*Subchronic and chronic toxicity*

The main goal of chronic fish tests is primarily to evaluate potential effects on fish reproduction and on offspring. Other regularly observed sublethal effects must be reported. These chronic tests are designed to determine a "No observed effect concentration" (NOEC) and a "Lowest observed effect concentration" (LOEC) (Turner 2003; OECD 1992, 2000).

Atrazine

The effects of subchronic exposure to atrazine on fish growth were investigated in zebrafish (*D. rerio*), with values for NOEC and LOEC determined to be 0.3 $\mu g\,L^{-1}$ and 0.9 $\mu g\,L^{-1}$, respectively, after 28 days of exposure (Plhalova *et al.* 2012a).

Simazine

Chronic toxic effects of simazine were observed on the growth of fry of fathead minnow (*Pimephales promelas*) and the NOEC and LOEC were reported to be 1.2 $mg\,L^{-1}$ and 2.5 $mg\,L^{-1}$, respectively (Turner 2003).

Terbutryn

A NOEC of 0.2 $mg\,L^{-1}$ and LOEC of 0.6 $mg\,L^{-1}$ of terbutryn were reported for zebrafish (*D. rerio*) in a 28-day trial (Plhalová *et al.* 2009). Considerably lower NOEC and LOEC values of 0.0007 and 0.005 $mg\,L^{-1}$, respectively, were reported for common carp (*C. carpio*) in a 36-day test on early life stages (Velisek *et al.* 2012b).

Terbuthylazine

The embryos and larvae of common carp (*C. carpio*) were investigated during a 30-day trial involving subchronic exposure to terbuthylazine, yielding a NOEC of 0.16 $mg\,L^{-1}$ and a LOEC of 0.52 $mg\,L^{-1}$ (Štěpánová *et al.* 2012a). A unique work describing the effects of terbuthylazine-2-hydroxy, the major degradation product of terbutryn and terbuthylazine, based its LOEC and NOEC values on inhibition of the growth of early life stages of common carp (*C. carpio*), determining them to be 0.002 $mg\,L^{-1}$ and 0.0001 $mg\,L^{-1}$, respectively, after 35 days of exposure (Velisek *et al.* 2014).

Metribuzin

Based on growth parameters, development, and histological examination, the LOEC value for metribuzin was reported to be 0.9 $mg\,L^{-1}$ for early life stages of common carp (*C. carpio*) exposed for 30 days (Štěpánová *et al.* 2012b). The chronic toxicity of metribuzin to zebrafish was investigated in a 28-day trial; in this juvenile growth toxicity test, the LOEC value was 33 $mg\,L^{-1}$ and the NOEC value was 16 $mg\,L^{-1}$ (Plhalova *et al.* 2012b).

*Triazine toxicity to freshwater invertebrates*

The most important parts of the aquatic food chain are freshwater invertebrates that serve as a food source for juvenile or adult fish. A reduction in a single species of zooplankton might affect the food supply for fish and thus the whole aquatic ecosystem (Turner 2003; Di Giulio & Hinton 2008).

Studies have revealed diverse sensitivities of aquatic invertebrates to triazines, indicating that triazines can be practically non-toxic, moderately toxic, or even highly toxic to these organisms (Fitzmayer *et al.* 1982; Wan *et al.* 2006). Water crustaceans, like water fleas (*Daphniidae*) or copepods (*Cyclopidae*), are generally good indicators of triazine herbicide contamination in the aquatic environment, because the acute toxicity concentration for this organism is mostly reported to be very low. In addition, simazine is considered to be acutely toxic (48-hour $LC_{50}$) to the water flea (*Daphnia magna*) at a concentration of 1.1 $mg\,L^{-1}$ (Johnson & Finley 1980); meanwhile, the maximum acceptable toxicant concentration (MATC) of atrazine for water fleas was estimated to be from 0.14 to 0.25 $mg\,L^{-1}$ (Macek *et al.* 1976). In a study with atrazine, the most sensitive invertebrates were reported to be midge larvae (*Chironomus tentans*) with a 48-hour $LC_{50}$ of 0.72 $mg\,L^{-1}$ (Macek *et al.* 1976). The results of acute toxicity studies on freshwater invertebrates with commercial triazine herbicides are shown in table 2 (Svobodova *et al.* 1987). Acute toxicity tests with atrazine on tropical freshwater cladoceran (*Pseudosida ramosa*) exhibited 48-hour $LC_{50}$ values of 20.9 $mg\,L^{-1}$ (Freitas & Rocha 2012). Signal crayfish (*Pacifastacus leniusculus*) was reported to be more sensitive than the fish for atrazine, hexazine, or metribuzin after 96-hours toxicity test with triazine pesticides (Velisek *et al.* 2013a). By contrast, triazine compounds are reported to be practically non-toxic to some invertebrates, with simazine being acutely toxic to glass shrimp (*Palaemonetes kadiakensis*) (Mayer *et al.* 1986) or crayfish (*Procambarus sp.*) (Turner 2003) at concentrations exceeding 100 $mg\,L^{-1}$.

**Tab. 1.** Acute toxicity of triazines to fish.

| Compound | Species | Scientific name | 96-hour $LC_{50}$ (mg $L^{-1}$) | Toxicity Category[a] | Reference |
|---|---|---|---|---|---|
| ATRAZINE | | | | | |
| n.s. | Crucian carp | *Carassius carassius* | >100 | practically non-toxic | Solomon *et al.* 2008 |
| 50% a.i. | Spotted snakehead | *Channa punctatus* | 42.380 | slightly toxic | Nwani *et al.* 2010b |
| n.s. | Common carp | *Cyprinus carpio* | 18.8 | slightly toxic | Neskovic *et al.* 1993 |
| n.s. | Sheepshead minnow | *Cyprinodon variegatus* | 16 | slightly toxic | Ward & Ballantine 1985 |
| n.s. | Coho salmon | *Oncorhynchus kisutch* | 15 | slightly toxic | Lorz *et al.* 1979 |
| 94% a.i. | Bluegill | *Lepomis macrochirus* | 8 | moderately toxic | Macek *et al.* 1976 |
| 94% a.i. | Brook trout | *Salvelinus fontinalis* | 6.3 | moderately toxic | Macek *et al.* 1976 |
| SIMAZINE | | | | | |
| 98.1% a.i. | Rainbow trout | *Oncorhynchus mykiss* | >100 | practically non-toxic | Johnson & Finley 1980 |
| tech. | Guppy | *Poecilia reticulata* | 49 | slightly toxic | Bathe *et al.* 1975 |
| 80% a.i. | Rainbow trout | *Oncorhynchus mykiss* | 40.5 | slightly toxic | Turner 2003 |
| 98.1% a.i. | Fathead minnow | *Pimephales promelas* | 5 | moderately toxic | Mayer *et al.* 1986 |
| PROMETRYNE | | | | | |
| (99% a.i.) | Bluegill sunfish | *Lepomis macrochirus* | 10 | slightly toxic | US EPA 1996 |
| n.s. | Zebrafish | *Danio rerio* | 3 | moderately toxic | Popova 1976 |
| n.s. | Commoncarp | *Cyprinus carpio* | 8–9 | moderately toxic | Popova ,1976 |
| 99% a.i. | Rainbow trout | *Oncorhynchus mykiss* | 2.9 | moderately toxic | US EPA 1996 |
| PROPAZINE | | | | | |
| n.s. | Goldfish | *Carassius auratus* | >32 | slightly toxic | Tomlin 1994 |
| n.s. | Rainbow trout | *Oncorhynchus mykiss* | 18 | slightly toxic | Beste 1983 |
| CYANAZINE | | | | | |
| n.s. | Sheepshead minnow | *Cyprinodon variegatus* | 18 | slightly toxic | Worthing & Walker 1987 |
| tech. | Fathead minnow | *Pimephales promelas* | 16.3 | slightly toxic | Johnson & Finley 1980 |
| 80% a.i. | Rainbow trout | *Oncorhynchus mykiss* | 9.00 | moderately toxic | Johnson & Finley 1980 |
| TERBUTRYN | | | | | |
| tech. | Zebrafish(juvenile) | *Danio rerio* | 5.71 | moderately toxic | Plhalová *et al.* 2010 |
| n.s. | Common carp | *Cyprinus carpio* | 4 | moderately toxic | Kidd & James 1991 |
| tech | Guppy(juvenile) | *Poecilia reticulate* | 2.85 | moderately toxic | Plhalová *et al.* 2010 |
| 59.4% a.i. | Sea bream | *Sparus aurata* | 1.41(72hLC50) | moderately toxic | Arufe *et al.* 2004 |
| n.s. | Rainbow trout | *Oncorhynchus mykiss* | 0.82 | highly toxic | Johnson & Finley 1980 |
| TERBUTHYLAZINE | | | | | |
| n.s. | Goldfish | *Carassius auratus* | 9.4 | moderately toxic | Hartley & Kidd 1987 |
| 98%% a.i. | Bluegill sunfish | *Lepomis macrochirus* | 7.5 | moderately toxic | US EPA 1995 |
| n.s. | Common carp | *Cyprinus carpio* | 4.7 | moderately toxic | Tomlin 1994 |
| 98%% a.i. | Rainbow trout | *Oncorhynchus mykiss* | 3.4 | moderately toxic | US EPA 1995 |
| METRIBUZIN | | | | | |
| 70% a.i. | Common carp | *Cyprinus carpio* | >100 | practically non-toxic | Velisek *et al.* 2009a |
| 70% a.i. | Rainbow trout | *Oncorhynchus mykiss* | 89.3 | slightly toxic | Velisek *et al.* 2008 |
| 95% a.i. | Bluegill sunfish(juvenile) | *Lepomis macrochirus* | 76 | slightly toxic | Fairchild & Sappington 2002 |
| 90% a.i. | Rainbow trout | *Oncorhynchus mykiss* | 42 | slightly toxic | US EPA 1998 |

## SYMPTOMS OF TRIAZINE POISONING

The effects of triazine herbicide exposure on the physiology of fish were evaluated by measuring a variety of indices. The assessment of behaviour, morphological status, biochemical and haematological parameters has become a valuable way of understanding normal and pathological processes and the toxicological impacts of exposure to triazines and their degradation products (Elia *et al.* 2002; Van der Oost *et al.* 2003; Velisek *et al.* 2011).

*Changes in fish behavior*

Fish display a variety of behavioral reactions to stress induced by many environmental factors. Such behavioral changes can induce negative effects at population or community levels, and, therefore, the behavioral responses of fish to triazine compounds is an important factor in environmental toxicology. In order to make an accurate assessment of toxicological hazards, monitoring must reflect an organism's behaviour, and the behavioral indices selected for monitoring must reflect natural fish physiology (Solomon *et al.* 2008; Amiard-Triquet 2009; He 2010; Amiard-Triquet & Amiard 2012).

Triazines are organic contaminants that are able to disrupt such complex behavioral patterns and, therefore, behaviour indicators may be considered as key markers of stress-related responses. For instance, there have been numerous studies in which behavior indicators were used to evaluate the impact of triazine on the fish organism. Rainbow trout exhibited reduced motility and balance disturbances after acute (96-hour) exposure to atrazine at 1.4 and 2.8 mg $L^{-1}$ (Fischer-Scherl *et al.* 1991). When exposed to various concentrations of atrazine (5–3125 µg $L^{-1}$), zebrafish significantly preferred dark habitats, and even the lowest concentration of atrazine (5 µg $L^{-1}$) significantly affected behavioral parameters (Steinberg *et al.* 1995). Exposure to atrazine at 0.5 µg $L^{-1}$ for 24 hours caused a significant increase in swimming burst activity in goldfish, and atrazine at 5 µg $L^{-1}$ induced various significant behavioral alterations in the fish – a decrease in grouping and an increase in the number of mouth openings at the surface (Saglio & Trijasse 1998). Similar clinical symptoms were reported in rainbow trout and common carp exposed to concentrations of metribuzin (62.51 mg $L^{-1}$ and 175.1 mg $L^{-1}$, respectively) for 96 hours; the following symptoms were described: increased respiration rate, loss of movement coordination, fish lying on their flanks and moving in this position (Velisek *et al.* 2008, 2009a). During 96 hours of exposure to atrazine (concentrations ranging from 25.0 to 53.0 mg $L^{-1}$), spotted snakehead (*Ch. punctatus*) were alert and displayed uncoordinated behavior such as restlessness, abnormal swimming behavior, vigorous jerks of the body, and loss of balance (Nwani *et al.* 2010b). Studies of migratory behaviour, which plays a key role in the biology of salmonids, have great importance in ecotoxicology. In a study with Atlantic salmon (*S. salar*), atrazine modified smolt swimming behavior and thus disrupted the downstream migration of the fish in fresh water and after sea water transfer (Waring & Moore 2004). Similarly, the exposure of Atlantic salmon (*S. salar)* smolts to atrazine at environmental concentrations (5 µg $L^{-1}$) significantly decreased the migratory activity of the fish (Moore *et al.* 2007).

By contrast, normal behavior with no signs of respiratory distress or any other alterations has been reported during experiments involving the chronic exposure of fish to triazines at real concentrations. The experimental exposure of common carp to simazine (45 µg $L^{-1}$) for 90 days did not result in any abnormalities with respect to swimming or respiratory behavior (Oropesa *et al.* 2009). Similarly, the exposure of common carp to subchronic or low concentrations of simazine (0.06, 4, 20, and 50 µg $L^{-1}$) or terbutryn (2, 20, and 40 µg $L^{-1}$) for 28 days did not induce significant behavioural abnormalities (Velisek *et al.* 2009b, 2010). Likewise, long-term exposures of common carp to terbutryn for 90 days (0.02, 0.2, and 2.0 µg $L^{-1}$) (Velisek *et al.* 2011), to simazine for 60 days (0.06 µg $L^{-1}$, 2 and 4 mg $L^{-1}$) (Stara *et al.* 2012), and to prometryn for 60 days (0.51, 8.0, and 80 µg $L^{-1}$) (Velisek *et al.* 2013b) induced no behavioral changes in the fish.

*Morphological changes*

In contrast to most biochemical and haematological parameters, morphological changes are higher-level responses of the fish organism to toxic compounds and generally indicate cellular damage (Van der Oost *et al.* 2003; Di Giulio & Hinton 2008). The histopathological examination of fish provides further information on

**Tab. 2.** Acute toxicity of triazines to freshwater invertebrates (48-hour LC50 mg $L^{-1}$) (Svobodova *et al.* 1987)

| Species | Scientific name | Commercial product and its active substances | | |
|---|---|---|---|---|
| | | **Zeazin S40** (atrazine 40%) | **Zeazin MIX** (atrazine 20%, prometryn 30%) | **Zeazin MIX A** (atrazine 30%, prometryn 20%) |
| Water flea | *Daphnia magna* | 48 | 36 | 34.4 |
| Copepods | *Cyclopidae* | 18 | 3.4 | 7.9 |
| Sludge worms | *Tubificidae* | 67 | 331 | 346 |

toxicopathological processes causing reversible or irreversible damage to organs and specific lesions. Histopathological approaches generally include examination of liver, kidney, gonads, or gills, and abnormalities can be used as biomarkers of aquatic pollution, in general, as well as of the impacts of triazine on fish, in particular (Hinton 1994; Van der Oost *et al.* 2003; Amiard-Triquet *et al.* 2012).

The effects of atrazine on fish have been very well documented and histopathological examination has played an important role in the assessment of the risks posed by such compounds to the aquatic environment. Alterations have mainly been observed in the liver, kidney and gills. While the exposure of rainbow trout (*O. mykiss*) to lower chronic atrazine concentrations (5, 10, 20, and 40 $\mu g\,L^{-1}$) for 28 days resulted in various alterations to renal corpuscles and tubules, with mitotic figures in the renal haemopoietic interstitium, acute atrazine (96 hours) exposure at sublethal concentrations (80 $\mu g\,L^{-1}$, and 1.4 and 2.8 $mg\,L^{-1}$) led to necrosis of fish renal cells (Fischer-Scherl *et al.* 1991). In common carp exposed to atrazine at a concentration of 1.5 $mg\,L^{-1}$ for 14 days, the most severe histopathological changes (hyperplasia) were found in the epithelial cells of gill (Neskovic *et al.* 1993). Chronic effects of atrazine were demonstrated in juvenile grey mullet (*Liza ramanda*), when atrazine affected some tissues in fish, mainly liver. After exposure of fish to atrazine at a concentration of 170 $\mu g\,L^{-1}$, the liver increased lipid inclusions with lipoid degeneration and reduced glycogen content. These changes were observed only during atrazine exposure; after the return of fish to clean water, enhancement of the hepatic metabolism occurred and the changes were reversed (Biagianti-Risbourg & Bastide 1995). Moderate structural changes in fish gills were observed after the acute 6-hour exposure of freshwater Peters' elephantnose fish (*Gnathonemus petersii*) to atrazine. Breaks in the gill epithelium appeared at a concentration of 0.50 $mg\,L^{-1}$ and these evolved into deep pits at 5.0 $mg\,L^{-1}$ (Alazemi *et al.* 1996). A juvenile growth toxicity test with atrazine was performed on zebrafish (*D. rerio*) and subsequent histopathological examination revealed alterations in the liver (dystrophic lesions of the hepatocytes) of fish exposed to a concentration of 90.0 $\mu g\,L^{-1}$ (Plhalova *et al.* 2012a). A similar effect on adult zebrafish females was observed after exposure to 10 and 1000 $\mu g\,L^{-1}$ of atrazine for 14 days. While the low dose induced only partial swelling of the hepatic cells, the high dose caused vacuolar degeneration in the hepatocytes (Jin *et al.* 2012). Atrazine affected the morphological structure of gills and liver of common carp during a 96-hour exposure experiment only at its highest tested concentration of 30 $mg\,L^{-1}$. Changes were manifested by severe multifocal lamellar teleangiectasis with dilation of the lamellar capillary on the gills and moderate to marked dystrophic lesions in the liver (Blahova *et al.* 2014).

Similar alterations to those induced in fish exposed to atrazine were described in fish after exposure to terbutryn, another triazine. Damaged liver was observed in the larvae of gilthead sea bream (*S. aurata*) after exposure to a commercial herbicide containing terbutryn (59.4%) and triasulfuron (0.6%). Histological examination of the liver of larvae exposed to a concentration of 2.5 $mg\,L^{-1}$ for 72 hours revealed a loss of cellular shape caused by the amount of lipid inclusions in the hepatocytes (Arufe *et al.* 2004). Sub-chronic exposure of common carp to terbutryn concentrations of 4, 20, and 40 $\mu g\,L^{-1}$ caused lipid inclusions in hepatocytes and the destruction of caudal kidney tubules after 28 days (Velisek *et al.* 2010). Early life stages of common carp were exposed to terbutryn at concentrations of 0.02, 0.2, and 2 $mg\,L^{-1}$ and showed alterations to the tubular system of the caudal kidney, but no histopathological changes were demonstrated in exposed fish gill (Velisek *et al.* 2012b). No histopathological alterations were found in the liver or cranial or caudal kidney tissue of one-year-old common carp after long-term exposure (90 days) to terbutryn at concentrations of 0.02, 0.2, and 2 $\mu g\,L^{-1}$ (Velisek *et al.* 2011). In an early-life stage fish toxicity test, destruction of the tubular epithelium and the disintegration of glomeruli were reported after the exposure of common carp to terbuthylazine-2-hydroxy, the main metabolite of terbutryn and terbuthylazine, for35 days. Alterations in the kidneys were observed in fish exposed to the highest tested concentrations of 1.4 $mg\,L^{-1}$ and 3.5 $mg\,L^{-1}$. However, no histopathological changes in liver or gills were recorded (Velisek *et al.* 2014).

A commercial herbicide with terbuthylazine as the active ingredient mainly affected the liver and gills of common carp exposed to a concentration of 3.3 $mg\,L^{-1}$. In this case, capillary aneurysms in gill filaments and local hyperplasia of the respiratory epithelium were found after 24 hours of treatment. However, all observed histopathological changes disappeared after a 6-day recovery period (Mikulikova *et al.* 2013).

Studies with simazine revealed similar effects on hepatocytes and renal tissue to hose induced by the previously mentioned triazines. The kidneys of common carp (*C. carpio*) were examined during a 90-day laboratory toxicity test with simazine at a concentration 45 $\mu g\,L^{-1}$. The most significant histopathological changes were globular eosinophilic foci and necrotic areas in the hematopoietic and excretory tissues. Alterations were apparent mostly between 45 and 75 days of exposure with the incidence of damage decreasing by the end of the test. Meanwhile, the livers of these simazine exposed fish exhibited necrotic areas and steatosis (Oropesa *et al.* 2009). Likewise, in a 28-day exposure study, simazine caused damage to hematopoietic tissue in the cranial kidney of one-year-old common carp at concentrations of 4, 20, and 50 $\mu g\,L^{-1}$ with a dose dependent effect. Increasing simazine concentrations also caused increasing histological changes (including

steatosis, hyperaemia, and necrosis) in livers of the same fish (Velisek *et al.* 2009b). In a similar long-term toxicity study involving the exposure of common carp to simazine concentrations of 0.06, 1, 2, and 4 mg $L^{-1}$ for 90 days, the main histological changes were hyaline degeneration of the epithelial cells of the renal tubules of the caudal kidneys; no other tissues were affected (Velisek *et al.* 2012a). Hyperplasia and slight necrosis of gill epithelial cells were also reported in common carp following subchronic exposure (90-day) to simazine at 45 µg $L^{-1}$ (Oropesa-Jiménez *et al.* 2005).

Like other triazines, prometryn caused steatosis in the hepatocytes of common carp and dose-dependent structural alterations in the tubular epithelium of carp caudal kidney, with the most prominent changes at concentrations of 8 µg $L^{-1}$ and 80 µg $L^{-1}$. This study did not reveal changes in other examined tissues (gill, spleen, or cranial kidney) (Velisek *et al.* 2013b).

The tested triazinone, metribuzin, induced similar morphological effects to other triazine compounds. A metribuzin-based commercial herbicide containing metribuzin at a concentration of 70% was tested on rainbow trout (*O. mykiss*) in an acute toxicity test lasting 96 hours. After exposure at a concentration of 62.51 mg $L^{-1}$, hyaline degenerations in renal tubules of the caudal kidney and the hydropic degeneration of liver cells around the central veins were observed. However, no additional histopathological changes were found in other tissues (skin, spleen, cranial kidney) (Velisek *et al.* 2008). Similarly, adult common carp (*C. carpio*) exhibited some degenerations and changes in renal tubules of the caudal kidney after 96 hours of exposure to metribuzin at 175.1 mg $L^{-1}$, but other examined tissues showed no histopathological alterations (Velisek *et al.* 2009a).

Finally, the toxic effects of triazine on gills are also highly significant for the fish organism and have been the subject of histopathological examination in many studies. Triazines are received by fish from the water environment through the gills (Gunkel 1981). The gills serve as the site for gas exchange, active and passive ionic transport, acid-base regulation, and nitrogenous waste excretion. Many environmental pollutants affect the morphological structure of the gill epithelium and thus they disturb physiological functions in the fish organism (Evans 1987). The toxic effects of triazine compounds were associated with moderate to severe morphological pathologies in exposed fish gill (Neskovic *et al.* 1993; Alazemi *et al.* 1996; Oropesa-Jiménez *et al.* 2005; Mikulikova *et al.* 2013; Blahova *et al.* 2014). In contrast, other studies with triazines did not reveal any histopathological alterations to examined gill tissue (Velisek *et al.* 2009a; Plhalova *et al.* 2012a; Velisek *et al.* 2012b; 2013; 2014).

Pathomorphological examination after acute exposure to some triazine pesticides revealed the increased presence of watery mucus on body surfaces or in transudate, i.e. extra vascular water in the body cavity. In rainbow trout (*O. mykiss*) fry, exposure resulted in the formation of a transudate which enlarged the body cavity and caused it to rupture (Svobodova *et al.* 1987). These characteristic findings were also observed after an acute (96-hour) toxicity test with metribuzin at 62.51 mg $L^{-1}$, when exposure resulted in the formation of transudate in the body cavity of rainbow trout. In addition, black pigmentation of the skin of exposed fish was also described (Velisek *et al.* 2008). Ninety-six hours of exposure to metribuzin at 175.1 mg $L^{-1}$ had the same effect on the common carp with the formation of transudate and dark skin (Velisek *et al.* 2009a). Both studies supposed that the transudation was a result of the leakage of proteins caused by damage to renal tubular epithelial cells, which was confirmed by the presence of plasma hypoproteinemia in both cases (Velisek *et al.* 2008; 2009a).

*Changes of haematological profile*

The assessment of haematological profiles in pesticide-exposed fish is required for an understanding of both normal and pathological processes and for the evaluation of toxicological risks. Haematological changes are usually the first detectable and quantifiable responses to environmental change; however, an appropriate biomarker must be chosen that is selective enough to provide information on the induction of stress caused by exposure to chemical pollutants (Van der Oost *et al.* 2003; Hawkins & Mawdesley 2006; Di Giulio & Hinton 2008; Velisek *et al.* 2009a; Hrubec & Smith 2010).

The procedures used to evaluate fish haematological profiles are based on the unified methods for haematological examination of fish and the associated parameters include erythrocyte count (RBC), haemoglobin concentration (Hb), haematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells count (WBC), leukocyte count (Leuko), and the differential leukocyte count (Leukogram) (Svobodova *et al.* 1991; Hawkins & Mawdesley 2006).

Fish exposed to triazine compounds exhibited various haematological changes, e.g. RBC count, PCV, Hb, MCV, MCH, MCHC, WBC (Neskovic *et al.* 1993; Velisek *et al.* 2009a, 2009b, 2010, 2011) and many previous studies have demonstrated the profound influence of triazines on the haematopoietic systems of fish – for example, when triazine herbicides caused the increased breakdown of red blood cells (Svobodova & Pecena 1988), or when atrazine, in particular, caused an increase in the mitotic index of cells in the renal haemopoietic interstitium of rainbow trout (*O. mykiss*) after lower chronic exposures at concentrations of 5, 10, 20, and 40 µg $L^{-1}$, or necrosis at concentrations of 80 µg $L^{-1}$, 1.4 and 2.8 mg $L^{-1}$ (Fischer-Scherl *et al.* 1991). A decrease in certain haematological indices (RBC, Hb, and PCV) was described in Nile tilapia (*O. niloticus*) and Golden Nile Catfish (*Chrysichthys auratus*)

exposed to atrazine at concentrations of 3 and 6 mg $L^{-1}$ (96% a.i.) (Hussein *et al.* 1996). The exposure of smolt to atrazine at 100 µg $L^{-1}$ in fresh water induced significant increases in the PCV of fish after seawater challenge in a study describing the effects of atrazine on the development of juvenile Atlantic salmon (*S. salar*) (Nieves-Puigdoller *et al.* 2007). Acute exposure to metribuzin (62.51 mg $L^{-1}$) resulted insignificantly lower values of RBC and PCV and significantly higher values of MCH in rainbow trout (*O. mykiss*) (Velisek *et al.* 2008); similarly, the acute exposure of common carp to metribuzin (175.1 mg $L^{-1}$) resulted in significantly reduced values of PCV, Hb, MCV, and WBC (Velisek *et al.* 2009a). Only a slight decrease in PCV was described in common carp exposed to 45 µg $L^{-1}$ of simazine for 90 days (Oropesa *et al.* 2009). Acute 96-hour exposure to terbuthylazine caused significant decreases in PCV, WBC, and lymphocyte count in common carp (Dobšíková *et al.* 2011). After the long-term (60-day) exposure of common carp to prometryn (8.0 and 80 µg $L^{-1}$), the values of MCH, Hb and MCHC were increased (Velisek *et al.* 2013b).

*Immunological changes*

Fish, naturally, come into direct contact with chemicals that are present in the aquatic environment. These chemicals, including ubiquitously occurring herbicides, can have undesirable adverse effects on important physiological mechanisms in fish that help protect them against disease, i.e. the fish immune system (Anderson 1996). Most published research papers assume that, in fish, triazine substances suppress both nonspecific and specific immune defense mechanisms.

Although one study revealed that the presence of atrazine in the diet of common carp after 84 days of feeding did not induce any changes in immunological parameters such as splenic index (Cossarini-Dunier *et al.* 1988), another study revealed that carp kept in water contaminated with atrazine at a concentration of 0.5–1 mg $L^{-1}$ exhibited a decline in non-specific immune defense (Svobodova & Pecena 1988). Similarly, changes in lymphocyte and neutrophil granulocyte counts were reported in carp following acute poisoning with commercial herbicides containing atrazine and other triazines (Svobodova *et al.* 1987). The response of the carp immune system to acute (96-hour) exposure to triazine was evaluated with atrazine at concentrations of 5, 15, 20, and 30 mg $L^{-1}$ with a following 7-day recovery period in pure water. A significant decrease in white blood cell counts was reported in all exposed fish; most noticeably, however, lymphocyte counts decreased significantly at atrazine concentrations of 15, 20, and 30 mg $L^{-1}$. However, increases in monocyte and segmented and band neutrophil granulocyte counts were described in almost all exposed fish. Alterations to fish immunity continued to be reported even during the recovery period in atrazine-free water (Blahova *et al.* 2014). A commercial triazine herbicide with terbuthylazine as the active ingredient at a concentration of 2.25 mg $L^{-1}$ was used in an acute toxicity test on common carp lasting 96 hours. The exposure caused a significant decrease in leukocyte and lymphocyte counts in treated carp (Dobšíková *et al.* 2011). Whereas sub-chronic exposure to terbutryn at concentrations of 0.02, 4, 20, and 40 µg $L^{-1}$ for 28 days induced no changes in leukocyte count or differential leukocyte count in exposed fish (Velisek *et al.* 2010), long-term exposure to terbutryn at concentrations of 0.2 and 2 µg $L^{-1}$ lasting 90 days induced a significant reduction in leukocyte and lymphocyte counts (Velisek *et al.* 2011). Changes in the immune system of fish were also reported after the subchronic and chronic exposure of common carp to simazine. While increased lymphocyte and decreased band neutrophil granulocyte counts, and development phase myeloid sequences were found in common carp exposed to concentrations of 4, 20 and 50 µg $L^{-1}$ for 28 days (Velisek *et al.* 2009b), long-term exposure of the same kind of fish to simazine at concentrations of 0.06, 1, 2, and 4 µg $L^{-1}$ showed only a significantly decreased leukocyte count after 90 days (Velisek *et al.* 2012a). The following haematological studies revealed a decline in nonspecific immunity in different fish after acute 96-hour toxicity tests with commercial herbicides containing metribuzin. The exposure of rainbow trout (*O. mykiss*) to a herbicide with metribuzin at a concentration of 62.5 mg $L^{-1}$ caused a significant decrease in lymphocyte count and a significant increase in both segmented and band neutrophil granulocyte counts (Velisek at al. 2008); another study reported a decreased leukocrit value and a decreased leukocyte count after the treatment of common carp with a commercial herbicide containing metribuzin at a concentration of 175.1 mg $L^{-1}$. These changes were also associated with increased monocytes, basophils, and neutrophil and granulocyte bands and segments (Velisek *et al.* 2009a).

*Biochemical indices*

It has been proposed that, in general, stress induced by some pesticides elevates the transamination pathway, which is likely to contribute to the toxic effects of these compounds (Philip & Rajasree 1996; Svoboda 2001; Velisek *et al.* 2011). Biological responses such as biochemical and haematological alterations are usually the first detectable and quantifiable responses in fish exposed to triazines. Several parameters of physiological responses in affected fish are deemed potential biomarkers of contamination, such as specific enzymes or metabolic products (Van der Oost *et al.* 2003; Amiard-Triquet *et al.* 2012). Biochemical examination of fish plasma usually involves measuring of glucose (GLU), total protein (TP), cholesterol (CHOL) or ammonia; and activity of selected enzymes, such as aspartate aminotransferase (AST) or alanine aminotransferase (ALT).

Increased levels of GLU and decreased levels of serum TP and CHOL were found in common carp after subchronic exposure (72 hours) to atrazine at a

concentration of 0.1 mg $L^{-1}$ (Gluth & Hanke 1985). An increase in ALT activity in rainbow trout after 14 days of exposure to atrazine at concentrations of between 1.5 and 6.0 mg $L^{-1}$ (Neskovic *et al.* 1993) and decreases in TP, ammonia and AST in the same fish after 96 hours of exposure to metribuzin at 62.51 mg $L^{-1}$ (Velisek *et al.* 2008) were reported. Some previous authors also reported a similar decrease in TP levels after acute exposure to atrazine in rainbow trout (atrazine concentration of 50 μg $L^{-1}$) (Davies *et al.* 1994) and in Nile tilapia (*O. niloticus*) and Golden Nile Catfish (*Ch. auratus*) (atrazine concentration of 3 mg $L^{-1}$) (Mekkawy *et al.* 1996). A significant decrease in the activity of AST and a significant increase in ammonia levels in common carp were found after subchronic exposure (28 days) to simazine at concentrations of 4, 20, and 50 μg $L^{-1}$ (Velisek *et al.* 2009b). Meanwhile, chronic exposure of common carp to simazine (90 days) at concentrations of 1, 2, and 4 mg $L^{-1}$ resulted in a significant decrease in plasma ALT activity compared with controls (Velisek *et al.* 2012a).

*Biotransformation indices*

The metabolism of most xenobiotic compounds involves conversion into a more water-soluble form, which can be excreted from the organism. This biotransformation is catalyzed by phase I and phase II enzymes and, generally, these enzymes may be induced or inhibited upon exposure of fish to xenobiotics (Bucheli & Fent 1995; Van der Oost *et al.* 2003).

For investigation of the phase I response, cytochrome (CYP) concentrations and ethoxyresorufin-O-deethylase (EROD) activity are commonly used. After subchronic exposure to metribuzin (Modra *et al.* 2008; Plhalova *et al.* 2012b), terbuthylazine (Mikulikova *et al.* 2011), and simazine (Velisek *et al.* 2012a), there was no evidence of the impact of triazines on the phase I detoxification system in exposed fish. By contrast, CYP content was induced by atrazine in common carp gills following a 40-dayexposure period (Fu *et al.* 2013). In addition, changes in EROD activity described in rainbow trout (*O. mykiss*) exposed to terbutryn and terbuthylazine were subject to thermal influence. The highest measured EROD activities in fish exposed to triazines occurred in a water temperature of 4°C, the activities decreasing with increasing water temperature (Tarja *et al.* 2003).

The main process of phase II involves the excretion of chemicals facilitated by conjugation reactions with compounds having more polar groups (e.g. glutathione GSH). The responses of the enzymes and cofactors of this second biotransformation phase are widely monitored via the measuring of glutathione S-transferase (GST) activity and glutathione (GSH) concentration. However, the GSH molecule itself is very closely linked with indices of oxidative stress and very often classified as an indicator of oxidative stress. The biomarkers of phase II may also be affected after fish exposure to triazines, but still relatively little is known about the enzyme response in piscine phase II systems (Van der Oost *et al.* 2003; Blanchette *et al.* 2007; Di Giulio & Hinton 2008; Slaninová *et al.* 2009). The liver is considered to be the primary organ for biotransformation and, therefore, has been widely studied. For example, alterations in GST activity were found in bluegill sunfish after exposure to atrazine: there was significantly increased activity in liver after exposure to concentrations of 6 and 9 mg $L^{-1}$, and also in gills after exposure to a concentration of 9 mg $L^{-1}$ (Elia *et al.* 2002). In contrast, long-term exposure to simazine at environmental concentrations did not influence liver GST activity, or GSH concentration in common carp (Velisek *et al.* 2012a). Changes in biotransformation enzymes have been registered even in early-life stages of fish after some levels of triazine exposure. Atrazine caused a decrease in GST activity in early life stages of zebrafish (Wiegand *et al.* 2000) and also decreased GST activity in juvenile zebrafish at the highest concentration of 90 μg $L^{-1}$ in a 28-day trial (Blahová *et al.* 2013). In addition, embryo-larval stages of common carp exposed to metribuzin for 30 days exhibited increased GST activity in all experimental groups (0.9, 4, 14, and 32 mg $L^{-1}$) (Hostovsky *et al.* 2012).

*Oxidative stress indices*

In fish, the toxic effects of most pesticides, including triazine herbicides, are associated with the induction of oxidative stress (Di Giulio & Hinton 2008; Slaninová *et al.* 2009; Haluzová *et al.* 2010). The enzymes of antioxidant defence and the products of free radical damage can be used as biomarkers of oxidative stress in fish, offering great promise with respect to providing additional information about the toxic effects of triazines (Van der Oost *et al.* 2003).

Some of the enzymes of antioxidant defences are investigated as biomarkers of oxidative stress. Superoxide dismutase (SOD) controls the level of superoxides by dismutation. Catalase (CAT) is known as enzyme catalyzes a dismutation reaction of hydrogen peroxide in organism, which is produced by SOD. It is large protein enzyme including four subunits each with a ferric hem group. Glutathione peroxidase (GPx) is responsible for detoxification of hydrogen peroxide by the oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG). It is selenium dependent enzyme and it can also reduce fatty acid peroxides (LOOH). With activity of GPx is related function of glutathione reductase (GR), which catalyzes the reduction of GSSG to reduce form GSH with NADPH providing electrons (Di Giulio & Hinton 2008; Slaninová *et al.* 2009).

Atrazine induced oxidative stress in liver samples from female zebrafish (*D. rerio*) when the activities of the antioxidant enzymes SOD and CAT increased in a dose dependent approach in the liver of female zebrafish after a 14-days atrazine exposure (Jin *et al.* 2010). After long-term terbutryn exposure (0.02, 0.2, and

2.0 μg $L^{-1}$), significantly lower GR activity was found in the liver of juvenile common carp in all experimental groups (Velisek *et al.* 2011), and exposure to atrazine in spotted snakehead (*Ch. punctatus*) resulted in a similar increase in antioxidant GR activity (Nwani *et al.* 2010a). The activity of the antioxidant enzymes SOD, CAT, and GPx in common carp (*C. carpio*) increased with higher concentrations of simazine (2 and 4 mg $L^{-1}$) after 14 and 28 days of exposure, but decreased after 60 days of exposure (Stara *et al.* 2012). Other authors demonstrated similar changes in oxidative stress indices, antioxidant defense systems, and enzyme activity in fish after chronic or subchronic exposure to triazine compounds (Zhang *et al.* 2004; Oropesa *et al.* 2009; Zhang *et al.* 2010). The exposure of early life stages of common carp to atrazine at concentration of 0.3 μg $L^{-1}$ resulted in significantly increased activities of GPx, GST, SOD, and CAT in 30 days toxicity test (Chromcová *et al.* 2013).

A number of biochemical indicators are associated with increased fluxes of oxy-radicals and some of the products related to oxidative stress damage serve as biomarkers of the effects of exposure to triazines. Lipid peroxidation (LPOX) is very well-known – in particular, the oxidation of polyunsaturated fatty acids, perhaps the most studied targets of free radicals induced by oxidative stress (Di Giulio & Hinton 2008). The evaluation of LPOX levels by means of the measurements of degradation products such as aldehydes, alkenals, hydroxynonenals, acetone, and malondialdehyde (MDA) is performed in many works describing the influence of triazine compounds on the induction of oxidative stress. The most commonly used method for evaluating LPOX is the spectrophotometric measurement of color adducts formed from the reaction of LPOX products, especially malondialdehyde, with 2-thiobarbituric acid. This method is known as the TBARS method – from Thiobarbituric Acid Reactive Substances (TBARS) (Lushchak 2011). After chronic exposure of juvenile common carp to simazine, no significant differences in TBARS levels in fish tissues compared to the control group were found (Stara *et al.* 2012). A significant increase in liver MDA level was revealed in bluegill sunfish exposed to atrazine for 96 hours at concentrations of 6 and 9 mg $L^{-1}$, while no response was found in the gill (Elia *et al.* 2002). Similarly, atrazine at concentrations of 100 and 1000 μg $L^{-1}$ induced a dose-dependent increase in MDA levels in the liver of female adult zebrafish after 14 days of exposure, but did not induce a significant increase in ovarian tissues (Jin *et al.* 2010). Atrazine at 10.6 mg $L^{-1}$ elevated the TBARS level in the liver of spotted snakehead (*Ch. punctatus*) after 15 days of exposure (Nwani *et al.* 2010a). The exposure of common carp to atrazine for 40 days resulted in a dose-dependent increase in MDA content in liver, gill, brain and kidney tissues, with the most notice able response in the liver at an atrazine concentration of 428 μg $L^{-1}$ (Xing *et al.* 2012a, 2012b). LPOX levels were also significantly higher in juvenile zebrafish after 28 days of atrazine exposure (90 μg $L^{-1}$) (Blahová *et al.* 2013). In contrast, simazine (0.06, 2, and 4 mg $L^{-1}$) did not influence LPOX levels significantly in common carp after a 60-day chronic toxicity test in which brain, gill, muscle, liver, and intestine tissues were investigated (Stara *et al.* 2012).

*Endocrine and reproductive disruption*

Although triazine herbicides are designed primarily to effect plant cells, they can still seriously disrupt processes in animal cells. One of these processes is disruption of the endocrine function of some organs and cells, which can disrupt the entire process of development, sex differentiation, and every aspect of reproduction. Endocrine disrupting compounds or chemicals are able to interfere with reproduction through mimicking or inhibiting the action of natural hormones and altering the normal regulatory function of the endocrine system (Keith 1998; Segner *et al.* 2006; Di Giulio & Hinton 2008). While many studies have predicted triazine herbicides as potential endocrine disruptors, most of them focusing on atrazine, data on the potential hormonal effects of triazines in fish are still controversial (Hayes 2004; Solomon *et al.* 2008; Hayes 2009; Hayes *et al.* 2011).

Atrazine exposure at concentrations of 100 and 1000 μg $L^{-1}$ had a relatively weak effect on sex steroid genesis and did not show any clear estrogenic effect in adult goldfish males; however, it influenced 17-estradiol and 11-ketotestosterone biosynthesis after 21 days of exposure. In addition, atrazine exposure also induced structural disruption in the testes of males and elevated levels of atresia in the ovaries of female goldfish (Spanò *et al.* 2004).

The exposure of zebrafish juveniles (*D. rerio*) to relevant environmental atrazine concentrations (in the range 1–100 μg $L^{-1}$) for 30 days did not confirm the effects of atrazine on aromatase gene expression, aromatase being the key steroidogenic enzyme responsible for the conversion of androgens to estrogens (Kazeto *et al.* 2004). In an acute toxicity test (72 hours), atrazine affected hormone signalling and endocrine gene expression in young zebrafish (*D. rerio*). Significant responses were observed with low, environmentally relevant atrazine concentrations, which elevated the fish gene expression encoding aromatase (2.2 μg $L^{-1}$) and increased the ratio of female to male fish (22 μg $L^{-1}$) (Suzawa & Ingraham 2008). In contrast, the activity and expression of aromatase in the gonads of juvenile female goldfish were investigated after exposure of the fish to atrazine, but no significant effects were detected, even after exposure at high concentrations (100 and 1000 μg $L^{-1}$) for 56 days (Nadzialek *et al.* 2008).

The impact of atrazine on the reproduction of fathead minnow was evidenced by a decrease in egg production and reductions in spawning events. Concentrations of atrazine close to environmental concentrations (0.5, 5.0, and 50 μg $L^{-1}$) had significant effects

on fish reproductive output, and gonad abnormalities were also observed in atrazine-exposed males and females of fathead minnows; however, no significant effect of atrazine was observed in steroid hormone concentrations or aromatase activities in either sex (Tillitt *et al.* 2010). In a similar study to the above, atrazine also had significant effects on the reproductive output of Japanese medaka (*Oryzias latipes*). Total egg production was lower in all atrazine-exposed fish groups (0.5, 5.0, and 50 $\mu g\,L^{-1}$) when compared to the controls. Although atrazine had no effect on aromatase content or 17-estradiol and testosterone levels after a 30-day-trial, medaka males exposed to atrazine had a greater number of abnormal germ cells (Papoulias *et al.* 2014). Simazine at concentrations of 0.1, 0.5, 1.0 and 2.0 $\mu g\,L^{-1}$ and atrazine at concentrations of 0.5 and 2.0 $\mu g\,L^{-1}$ affected the reproductive priming effect by reducing the milt in mature male salmon parr (*S. salar*). Even though simazine and atrazine individually reduced the response of males to the pheromones, the mixture of both pesticides had no synergic effect; i.e. the effect was similar to that of each individual compound (Moore & Lower 2001). Atrazine also had a profound effect on the reproduction of the sea water fish red drum (*Sciaenops ocellatus*), when larvae exposed to atrazine at 40 and 80 $\mu g\,L^{-1}$ for 96 hours showed physiological and behavioral alterations compromising their survival potential (Alvarez & Fuiman 2005). Short-term exposure to environmentally realistic concentration (5 $\mu g\,L^{-1}$) of atrazine had an effect on mummichog (*Fundulus heteroclitus*) offspring via a negative effect on larvae osmotic control (Fortin *et al.* 2008).

## CONCLUSIONS

Anthropogenic activities, especially agriculture, are sources of pesticide input into the aquatic environment; triazine contamination is linked to extensive pesticide application, domestic and urban wastewater effluents, and diffuse sources connected with industrial production. In most studies, reported triazine concentrations in waters do not exceed the level of micrograms per litre and these have usually been regarded to be of ecologically low risk.

Many studies have dealt with the assessment of the acute or chronic toxicity of triazine-based compounds and their effects on aquatic animals. Although these studies indicated that triazines are generally toxic to various fish species at an acute level, the chronic effects of sublethal or real environmental concentrations of these compounds have not been fully investigated or considered. On the basis of toxicity data on the chemicals in question, triazines were found to be moderately or even highly toxic to fish, which makes fish a suitable non-target organism for ecotoxicological risk evaluation. In addition, such compounds have negative impacts on aquatic invertebrates. The results of acute, subchronic, or chronic toxicity studies with freshwater invertebrates indicate the relevant toxic effects of triazines on freshwater invertebrates but such effects also depend on many environmental factors and the compositions and structures of the chemicals themselves. It appears that daphnids (*Daphnidae*) and copepods (*Cyclopidae*) are the most sensitive to the majority of triazine compounds that were tested, either as pure substances or active ingredients, therefore, may serve as sensitive non-target biomarkers of triazine herbicide contamination in the environment.

Most of the mentioned studies have confirmed stress to be induced by the presence of triazines in the aquatic environment and that behavior changes are indicators of physiological responses in fish. Although some triazines did not affect fish behavior, especially in long-term exposures to low concentrations, impacts on the overall behavioral responses of fish cannot be excluded. Burst swimming reactions are an integral part of the alarm reaction in fish as a response to stress, and these behavioral disturbances have their origins in various biochemical and physiological pathways. Reduced swimming capability, as well as other alterations in fish motion and migration, may reduce the ability of fish to escape from predators or obtain food, while an effect on their reproductive behavior may result in a decrease in wild species populations.

As previous authors concluded, triazine pesticides have direct effects on fish, mainly on kidney structure and function (Oropesa 2009; Velisek *et al.* 2008, 2009a), but also cause various kinds of degeneration and histopathological changes in the liver (Biagianti-Risbourg & Bastide 1995; Arufe *et al.* 2004; Oropesa *et al.* 2009; Velisek *et al.* 2009b). The kidney is an important organ for the stability and maintenance of a fish's internal environment with respect to water and salt, excretion, and ion exchange, and also, partially, for the metabolism and excretion of xenobiotics. Triazine compounds disrupt the morphological structure of the kidney, even under long-term exposure at low environmental concentrations. Such nephrotoxic stress caused by exposure to these aquatic pollutants presents a serious threat to all the physiological functions that the kidneys help to maintain (Oropesa *et al.* 2009; Velisek *et al.* 2014). Similarly, the liver plays an essential role in the fish metabolism of xenobiotics; the hepatocytes are the main locations for catabolic and anabolic processes and liver tissue provides an energy reserve to assist fish survival. The cellular response of gills to triazine exposure could indicate adaptation or harmful processes. If triazines are present long term and occur widely in the aquatic environment, the gills are the organs most likely to undergo significant or continual exposure. In addition, another characteristic finding after triazine exposure was transudate in fish body cavity. This resulted from the damage of epithelial cells of renal tubules and hypoproteinaemia. However, in some studies, some changes were shown to be moderate and indicative of an adaptation response in fish to low triazine concen-

trations. Generally it can be concluded that all alterations and changes in affected fish organs, especially degenerations, slow down or completely block the metabolic pathways of the fish, and, thus, the chronic or sub-chronic exposure of fish to triazines can have a profound impact on both the bodies of individuals and whole populations. Thus, histopathological examinations of triazine poisoned fish provide reliable data for the monitoring of toxic effects on fish organs.

Usually, haematological parameters are not considered as specific biomarkers with respect to their responses towards chemical stressors; however, haematological profiles of blood can provide important information about the internal environment of the organism and the general physiology and health status of the exposed fish (Van der Oost *et al.* 2003; Hrubec & Smith 2010). In some studies, the most commonly altered haematological indices were haemoglobin concentration and haematocrit. These alterations have often been interpreted as the result of acute stress mediated by catecholamines (Tort *et al.* 2002) with immature erythrocytes being released from the spleen. Haemodilution following damage to fish organs or as a compensatory response in fish to maintain gas transfer after respiratory stress is also cited (Velisek *et al.* 2008) as well as an increase in haematocrit caused by splenic contraction after the general impact of stress (Hrubec & Smith 2010).

White blood cells play a critical role in both the non-specific and specific defense mechanisms of adaptive immunity. In fishes, stress often leads to leukopaenia marked as lymphopaenia and neutrophilia (Hrubec & Smith 2010). The effects of triazine herbicides on the fish immune system are often manifested by changes in white blood cell counts in exposed fish. The reduction in both leukocyte and lymphocyte counts indicates a decrease in the non-specific immunity of fish. This demonstrates that triazines seriously affect and threaten the immune response of fish (Dunier 1996; Austin 1999). Therefore, they are hazardous environmental contaminants which can cause immunosuppression in fish, leading to the outbreak of disease and infection.

The leakage of enzymes into the blood is frequently indicative of cellular membrane damage in affected organs. After triazine exposure, the most cited changes in fish blood biochemical parameters are in the activity of transferases (alanine or aspartate aminotransferase) along with alterations in ammonia level and decreased protein concentrations, which together indicate liver damage or suspected hepatotoxic consequences. Changes in other plasma biochemical parameters, such as other enzymes activities or the level of metabolic intermediates (e.g. glucose, cholesterol), are used as relevant stress indicators (Davies *et al.* 1994; Oropesa *et al.* 2009; Velisek *et al.* 2012).

According to studies using biotransformation enzymes to estimate the effects of triazines on fish, total cytochrome concentration or ethoxyresorufin-O-deethylase activity do not seem to be reliable biomarkers of triazine contamination in fish. However, commonly used measurements of alterations in glutathione S-transferase activity or glutathione concentration appear to reliably reflect the pollution of the fish environment by some triazine herbicides (Van der Oost *et al.* 2003; Hostovsky *et al.* 2012; Blahová *et al.* 2013).

The response of the fish organism to triazine exposure in terms of the activities of antioxidant enzymes could be an adaptive response to protect the fish from triazine-induced oxidative stress; however, further research is required to establish the significance of these enzymes with respect to their potential use in risk assessment and measurement single enzymes of antioxidant defences are not specific or sensitive enough for investigation of pesticides influence. An increase or a decrease of activity can be connected with changes of sex and reproductive condition, temperature, diet, dissolved oxygen, salinity, seasonal effects and physiological adaptation of fishes (Van der Oost *et al.* 2003). Lipid peroxidation levels may give specific information about the status of antioxidant defence and the liver is viewed as playing a key role in the assessment of the impacts of triazines (Van der Oost *et al.* 2003; Di Giulio & Hinton 2008; Slaninová *et al.* 2009).

On the basis of studies concerning the effect of triazines on endocrine function, it could be predicted that relatively low doses of atrazine might negatively influence normal hormone signalling in the fish organism and that, ultimately, triazine compounds affect fish reproduction itself. Morphological and physiological changes in gonads lead to reduced potency, and the effect of endocrine disruption is enhanced by disturbance of the metabolic pathways of steroid sex hormones. Triazine-induced changes in fish reproduction behaviour and the effects of triazine on larvae development may both influence fish populations in the wild or in aquaculture (Keith 1998; Segner *et al.* 2006).

The aquatic environment has been heavily contaminated with pesticides for many years. The benefits of triazine herbicides for agriculture and industry have made them one of the most used groups of herbicides. Due to the aforementioned facts, these contaminants represent a serious threat to the fish and aquatic environment. The risks presented by exposure to triazines at higher concentrations increase in some small watersheds and after storm runoff in areas with extensive agricultural activity associated with routine pesticide use. The persistence of triazine compounds and the long-term exposure of fish to them could have a profound influence on fish development. The amount of monitoring data available for atrazine is much greater than that for any other triazines, and although it has been restricted or banned, its persistence in the aquatic environment causes it to remain a relevant environmental pollutant. Most of this review has confirmed that triazine can induce morphological, biochemical, and physiological alterations in fish. Furthermore, it is generally consid-

ered that every negative effect on fish physiology, morphology, behaviour, or reproductive success may result in irreversible changes. Therefore, the overall impact of these changes on fish is still at odds with the generally positive view of triazine herbicides in ecotoxicological risk assessment. Another additional complication in attempting to make a comprehensive assessment of all environmental risks is that the aquatic environment is polluted by many other different substances and that synergistic or antagonistic effects can completely affect the response offish after exposure to such a chemical soup.

According to the results of many studies that have investigated the exposure of fish to triazine compounds, even at environmental concentrations, there remains a lack of consistency regarding the reported effects of triazines on fish and, therefore, further detailed research is required. The final question is whether the benefits of triazine herbicides as highly efficient weed agents outweigh their known and potential risks to fish and the aquatic environment.

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