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REVIEW ARTICLE

Chloro-s-triazines-toxicokinetic, Toxicodynamic, Human Exposure, and Regulatory Considerations

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Abstract: Chloro-s-triazines-atrazine, cyanazine, propazine, simazine, and terbuthylazine-are structurally similar herbicides, differing only in specific s-triazine4-and 6-N alkyl substituents. It is generally regarded that their toxicokinetics, such as, metabolic pathways, biological effects and toxicities, also share more similar features than the differences. Consequently, it is useful to compare their characteristics to potentially find useful structure-activity relationships or other similarities or differences regarding different active compounds, their metabolites, and biological effects including toxic outcomes. The ultimate goal of these exercises is to apply the summarized knowledge-as far as it is possible regarding a patchy and often inadequate database-to cross the *in vitro-in vivo* and animal-human borders and integrate the available data to enhance toxicological risk assessment for the benefit of humans and ecosystems.

Keywords: Chloro-s-triazines, biotransformation, metabolites, toxicity, human exposure, regulatory guidance.

1. INTRODUCTION

Chloro-s-triazines is a group of structurally similar herbicides differing only in specific s-triazine 4- and 6-N-alkyl substituents and sharing a common mechanism of action by inhibiting mitochondrial respiration [1-3]. Currently, atrazine, cyanazine, propazine, simazine, and terbuthylazine are in agricultural use. Atrazine was the most heavily applied pesticide in the USA in 1997 and is currently the second most abundantly applied pesticide [4], whereas terbuthylazine is the major triazine herbicide in the EU. In Table **1**, the chemical structures and some information about the usage history of each member as well as their main adverse effects in regulatory toxicological studies are presented.

Atrazine has been the focus of extended concern and research in the USA. Together with other triazine herbicides, it has been used for a long time in the USA and assessed by the US Environmental Protection Agency (EPA), the latest assessment was conducted in 2019 [5]. Terbuthylazine has been on the agenda of the European Food Safety Authority (EFSA) recently because of certain metabolites or degradation products in the drinking water, which has resulted in an EFSA Panel on Plant Protection Products and their Residues (PPR) Scientific Opinion [6].

Despite several risk assessment processes in the appropriate agencies and their public reports, there is a need for a more general review of triazine herbicides. The current review will address some special aspects regarding ADME (absorption, distribution, metabolism and excretion), with a focus on metabolism, toxicokinetics, and their potential significance in adverse effects in animals and humans. The review also provides information on regulatory guidance on permissible human exposures *via* various routes. Finally, some observations regarding the interpretations of toxicokinetic behavior of chloro-s-triazines in terms of animal and human toxicity and epidemiology will be presented.

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2. TOXICOKINETICS OF CHLORO-S-TRIAZINES

2.1. Regulatory Animal Studies on Toxicokinetics of Chloro-s-triazines

For obvious reasons, there are no human toxicokinetic studies performed on triazine herbicides, and regulatory animal (rat) studies are the principal source of toxicokinetic knowledge. Basic information on the extent of absorption, distribution, metabolism, and excretion of the radioactivity, usually by 24-hr spacing of measurements, is presented in Table **2**. Because salient toxicokinetic studies are mostly regulatory, they are based on the use of radioactively labelled compounds and mass balance considerations. More detailed toxicokinetic information is not often available, due to a study design focused on the elucidation of mass balance, or an elementary parent and metabolite separation as a function of time. Consequently, it is seldom possible to decipher more precise toxicokinetic data, such as clearance or half-life of the parent or major metabolites. This would be useful for the integration and interpretation of species differences or extrapolation to the human situation in the context of regulatory risk assessment [7, 8].

However, it is possible to conclude that regarding toxicokinetics, chloro-s-triazines are generally more similar; they are rapidly absorbed and distributed, with some, but not extraordinary, predominance to liver and kidney, extensively biotransformed, and excreted *via* urine and feces. Bile is an important primary excretory route for the studied compounds, pointing to a role for enterohepatic circulation.

2.2. Metabolic Pathways

Mammalian and vertebrate metabolism of chloro-s-triazines has been mostly studied in human or animal liver microsomes or S9 fractions [9-11]; few studies are available for *in vivo* animal studies

Table 1. Structures of chlorotriazine herbicides.

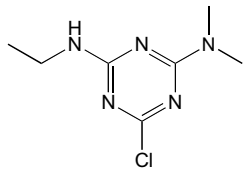
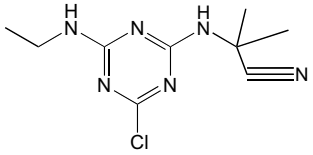
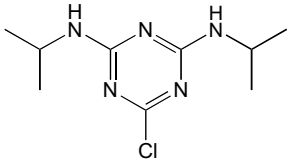
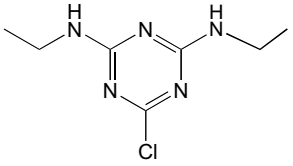
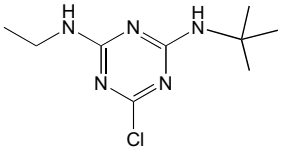
Compound and History of Use	Structure	Main Toxicities in Regulatory Studies
Atrazine (Currently used in USA since 1958 and Australia, but banned in EU since 2004)		Reduced BWG in mice and rats; increased liver weight genotox: no evidence teratogen: no evidence Repro & Develop toxicity: neuro-endocrine effects based on CNS toxicity carc: mammary gland tumors due to hormonal mechanism
Cyanazine (Currently used in USA since 1971, but not approved in EU since 2002)		Reduced BWG in mice and rats; increased liver, kidney weight genotox: equivocal teratogen in rats Repro & Develop toxicity: neuro-endocrine effects based on CNS toxicity carc: mammary gland tumors due to hormonal mechanism
Propazine (Currently used in USA since 1998)		Reduced BWG in mice and rats; increased liver weight genotox: no evidence teratogen: no evidence Repro & Develop toxicity: neuro-endocrine effects based on CNS toxicity carc: no evidence
Simazine (Currently used in USA since 1958)		Reduced BWG in mice and rats; increased liver weight genotox: no evidence teratogen: no evidence Repro & Develop toxicity: neuro-endocrine effects based on CNS toxicity carc; no evidence
Terbuthylazine (since 1983 in EU countries and elsewhere; less in USA)		Reduced BWG in mice and rats; increased liver weight genotox: no evidence teratogen: no evidence Repro & Develop toxicity: neuro-endocrine effects based on CNS toxicity carc: no evidence

Table 2. Some basic toxicokinetic characteristics of chlorotriazines. The data has been collected from assessment and regulatory documents of major regulatory agents and the WHO.

-	Atrazine	Cyanazine	Propazine	Simazine	Terbuthylazine
Absorption	Rapid, almost complete	Rapid	Rapid	Rapid	Rapid, extent >60%
Distribution	Erythrocytes, liver, kidney	No specific organ or tissue	No specific organ or tissue	Spleen, liver, kidney	Kidney, liver, blood
Metabolism	Extensive	Extensive	Extensive	Extensive	Extensive
Excretion	>50% in 24 hr urine>feces	-	82-95% in 48 hr urine>feces	Urine>faeces	75% in 24 hr, bile>urine>feces

[12, 13]. Regulatory dossiers are another source of appropriate information, which could be employed in the assessment. In general, metabolism proceeds *via* a few common primary pathways - principally N-de-alkylation (ethyl, isopropyl, t-buthyl), hydroxylation and subsequent carboxylation of alkyl groups, and dechlorination, which in mammals is suggested to proceed *via* non-enzymatic glutathione trapping [11] and subsequent mercapturate formation [12, 13]. Also, N-oxidations and imine formation at the N-alkyl group and subsequent glutathione conjugation (or trapping) have been detected [11], suggesting the production of reactive metabolites. Because parent compounds vary by only one or two alkyl groups, their removal by de-alkylation often results in common metabolites for different s-triazines. Metabolites in urine and bile (feces) are generally the same as *in vitro* systems, but quantitative differences abound. For example, conjugates with glucuronic acid (from UDPGA), sulphonic acid (from PAPS), or downstream mercapturates derived from glutathione conjugates are usually more abundant *in vivo* (based on available dossiers of Oxon and Syngenta). Interestingly, desethylterbuthylazine was detected in 100 % of urban wastewater samples from 6 Italian cities at concentrations up to 20 ng/L [14].

A general scheme of metabolic pathways for chloro-s-triazines is shown in Fig. (**1**). Detected metabolic pathways (and metabolites) for each chloro-s-triazine are listed in Table **3**. However, it has to be stressed that data are often patchy and incomplete, studies have often been performed with analytical tools that did not allow for a

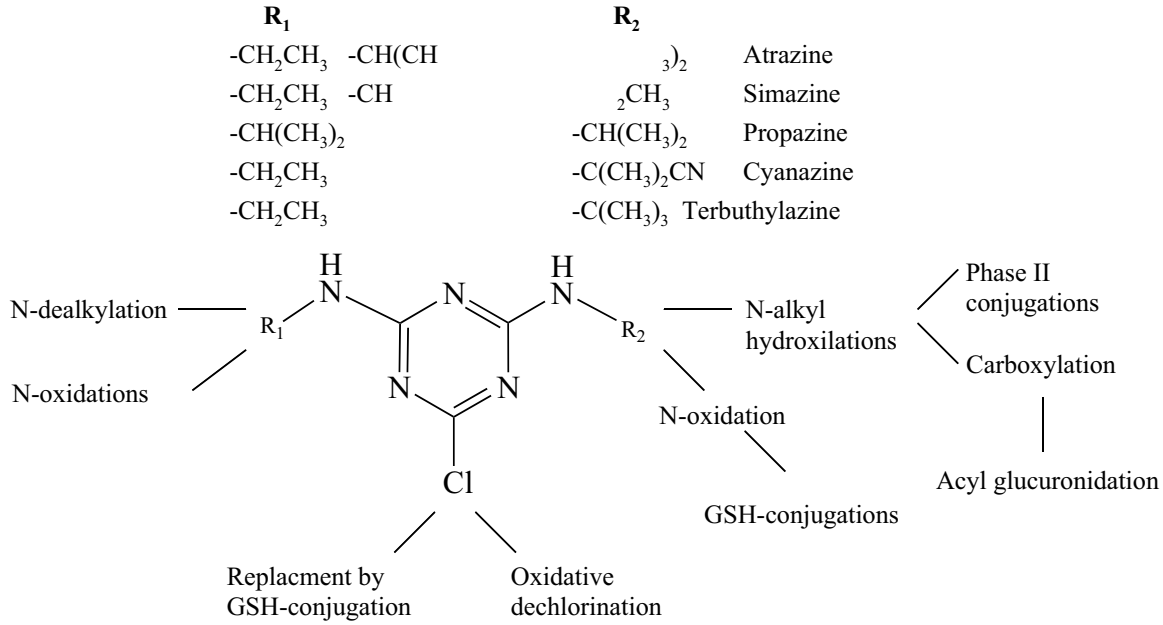


Fig. (1). Consensus metabolic map of chloro-s-triazines in mammals (modified from EFSA PPR Panel [6]).

Table 3. Metabolic pathways and metabolites in mammals, and soil and groundwater metabolites and degradants (data and primary sources from the National Data Base (https://pubchem.ncbi.nlm.nih.gov/search/) and (https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/).

-	Atrazine	Cyanazine	Propazine	Simazine	Terbuthylazine
Metabolic pathways in mammals	N-deethylation N-deisopropylation Oxidative dechlorination N-alkyl hydroxylation and glucuronidation Glutathione conjugation	N-deethylation Oxidative dechlorination Glutathione conjugation Cyano hydrolysis to amide and carboxylic acid	N-deisopropylation Di-N-dealkylation (→diamino-p) Glutathione conjugation	N-deethylation Di-N-deethylation Oxidative Dehalogenation Glutathione conjugation	N-deethylation N-de-t-buthylation Oxidative dehalogenation N-alkyl hydroxylation and glucuronidation Glutathione conjugation
Soil and/or Groundwater metabolites	Major: N-deethylatrazine N-deisopropylatrazine diaminochloro-atrazine hydroxyatrazine	Major: cyanazine acid cyanazine amide deethylcyanazine deethylcyanazine acid [15, 16]	Major: hydroxypropazine	Major: hydroxysimazine N-dealkylsimazine	Major: hydroxyterbuthylazine N-t-buthyl-ammelide Minor: desethylterbuthylazin, hydroxydesethylterbuthylazine cyanuric acid [16]

"complete" elucidation of metabolite spectrum, and thus gaps in knowledge are frequent. Also, major metabolites in soil and/or groundwater are listed because they are of primary importance for ecotoxicological considerations as well as for potential contamination of drinking water and consequent exposure of humans.

2.3. Metabolites in Groundwater

It is important to note that metabolites in groundwater are derived from either non-biological degradation or soil microbial catalysis; therefore, the spectrum of metabolites/degradants may considerably be different from mammalian metabolites, *i.e.* there are similarities, but also conspicuous differences in the spectrum or the amounts of different metabolites. The literature contains a large number of experimental studies on the formation of metabolites by the catalysis of specific micro-organisms or by certain natural or modified experimental soils [17]. Oxidative dichlorination and dealkylations as well as oxidative deamination predominate as microbial metabolic pathways, and ultimately the whole triazine molecule is completely degraded. When these metabolites expose humans (or animals), usually *via* drinking water, again a different spectrum of metabolites may be expected depending on the extent of triazine degradation in the environment. However, the metabolism and kinetics of the metabolites found in groundwater are usually not investigated in human *in vitro* systems or in experimental animals, at least not to a sufficient extent. In cases of major mammalian metabolites, usually more information on toxicokinetics is available.

2.4. Metabolizing Enzymes

It is generally known that cytochrome P450 (CYP) enzymes are the principal catalysts of phase I reactions (oxidation, reduction,

hydrolysis) on xenobiotic substances. Among triazines, atrazine is the most thoroughly studied; its N-deethylation is catalyzed by CYP1A1, 1A2, 2C19, 2D6, and its 3A4 and N-deisopropylation are catalyzed by all the previous as well as CYP2B6, CYP2C8, CYP2C9, and CYP2E1 [10, 12]. Published studies are available also for terbuthylazine; its N-deethylation is catalyzed by CYP1A1, 1A2, and 3A4 [10]. No published information is available for other chloro-s-triazines, but it is expected that at least N-dealkylation is catalyzed by some members of the above-mentioned CYP enzymes. Terbutryne and ametryne, derivatives of the s-triazine structure, are sulphoxidated by CYP2B6. *In vitro* studies using liver microsomes from rat demonstrated that CYP1A1/2, CYP2B1/2, CYP2C11, CYP2D1, and CYP2E1 are involved in atrazine metabolism, with CYP2B1/2 being implicated as the predominant enzyme [18-20].

As background information for risk assessment when comparing human and rodent metabolism, it would be of interest to identify specific rodent CYP enzymes participating in the metabolism of chloro-s-triazines. However, there seem to be no definitive studies on this topic.

Among phase II metabolizing enzymes, especially glutathione S-transferases (GSTs), UDP-glucuronosyl transferases (UGTs) participate in producing glutathione- and glucuronosyl-conjugates of chloro-s-triazines, although the assignment of the formation to specific enzymes is not regularly investigated. *In vitro* studies of phase II metabolism of atrazine in rat [21] and human liver fractions [22, 23] indicate that GSH conjugation is associated with glutathione-s-transferase pi (GST P) activity. GST P is increased in the rat [24, 25] and mouse [26] following treatment with atrazine.

2.5. Species Differences

Species differences in *in vitro* metabolism of chloro-s-triazines have been investigated in hepatic microsomes from rats, pigs, and humans. Principal phase I reactions were N-monodealkylation, hydroxylation of the isopropyl or tert-butyl moiety, and sulfoxidation of the substrates in all species. In general, all species produced the same types of metabolites, but with species-specific differences in the ratios of the metabolites [9]. Furthermore, extensive metabolism of chloro-s-triazines has been observed in *in vivo* studies in experimental animals, the major biotransformations involving N-dealkylation of the side-chains [27].

In humans, some specific metabolites have been identified in bodily fluids as biomarkers in estimating exposure. Although, dealkylated metabolites can also be formed in the environment and dominate in environmental exposures, diaminochlorotriazine and desethylatrazine were identified as the major human metabolites of occupational exposure to atrazine [28]. Dealkylated metabolites of triazine herbicides can be formed and excreted in the urine. These metabolites are not specific for a single triazine but provide class exposure information. Triazines can also be measured as the intact pesticide in blood products [29].

2.6. Methylthio-s-triazines

Terbutryn and ametryn are s-triazine herbicides and biocides, which differ from terbuthylazine and atrazine only by the replacement of chlorine with a methyl-sulphinyl group. The removal of the methylthio by hydroxylation leads to metabolic and degradation pathways, which are similar to those of terbuthylazine and atrazine. However, it seems that the principal reaction of the methylthio group is sulphoxidation by mammalian *in vitro* hepatic systems; no hydroxylation was detected *in vitro* [9], but some hydroxyterbutryn was found *in vivo* in rats and goats [30]. Sulphoxidation is catalyzed in *in vitro* systems principally by CYP1A2 [31]. More distal metabolites found in rat and goat urine are S-glucuronides and N-alkyl-O-glucuronides [30]. It has been demonstrated that in aquatic soil conditions, hydroxyterbutryn is the principal metabolite [32].

2.7. Significance of Dechlorination

In many mammalian *in vivo* or *in vitro* studies, dechlorinated metabolites, *i.e.* chlorine replacement by an hydroxyl group, have been found. It has been demonstrated that dechlorination is catalyzed principally by CYP enzymes in human tissues [33, 34]. However, *in vitro* metabolism studies specifically on atrazine metabolism in human liver preparations suggest that the presence of hydroxyatrazine is detected either as an impurity or is produced during the sample preparation [11, 12].

In soil, microorganisms contain specific enzymes capable of catalyzing the production of hydroxyl derivatives from various chlorinated triazines (*e.g.* Jurina *et al*. [16]). However, it is possible that at least some hydroxytriazines (or a certain portion of them) are genuine mammalian biotransformation metabolites, although at present their origin is uncertain.

A hypothetical origin of dechlorinatedtriazines could be associated with enterohepatic circulation of metabolites. Because several more distal metabolites of chloro-s-triazines such as conjugates are excreted in bile into the intestines, they come into contact with intestinal microflora. After appropriate dehalogenation, enzymes might de-conjugate the primary metabolites, which may be absorbed back into the body.

3. TOXICOKINETICS OF CHLORO-S-TRIAZINES

3.1. Inhibition and Induction of Xenobiotic-metabolizing Enzymes by Chloro-s-triazines

Obviously, being a substrate for a particular enzyme means that a substrate could serve at least as a competitive inhibitor for other substrates. Additionally, a substrate may also cause a mechanism-based and time-dependent inhibition based on the production of a tightly-bound metabolite, resulting in a long-lasting inhibition of the same enzyme or another enzyme. The only study in the published literature is by Abass *et al*. [35], demonstrating that atrazine was a moderate inhibitor of CYP3A4 at a concentration of about 3 μM; other CYPs enzymes were inhibited at much higher (>80 μM) concentrations or not at all.

Pretreatment of rats with high doses of atrazine or simazine both induced and depressed CYP-dependent enzyme activities *in vitro* [18]. A rat microsomal study on potential induction of P450 enzymes by 4 triazines has been reported; both induction and repression were found. Abass *et al*. [36] studied the induction of CYP1A2, CYP2A6, CYP2B6 and CYP3A4 model activity and mRNA levels in the human hepatoma cell line HepaRG after 24-hrincubation with DMSO, model inducers and atrazine. The cells were treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (arylhydrocarbon receptor (AHR) agonist and CYP1A2 model inducer), phenobarbital (PB) (CAR activator and CYP2A6 and CYP2B6 inducer), rifampicin (RF) (PXR agonist and CYP3A4 inducer), or DMSO (control cells). Atrazine induced mRNA and enzyme activity levels of several human CYPs in human hepatoma-derived HepaRG cells (Fig. **2**). Comparison with model inducers indicated that TCDD as a CYP1A inducer was vastly superior to atrazine, whereas atrazine caused a slightly greater induction of CYP2B6 than phenobarbital. AHR-controlled CYP1A was not inducible at the mRNA or activity level in the RTG-2 cell line by atrazine, prometryn, propazine or simazine [37].

Pre-treatment of rats with model inducers indicated that dealkylations of simazine, atrazine and propazine in liver microsomes were variably induced by 3-methylcholanthrene, phenobarbital, pyridine, and clofibrate, but not by dexamethasone [18].

Treatment of female rat with 50 or 100 mg/kg/day atrazine for 8 days showed induction of mRNA levels of 19 of 45 CYPs. GST expression, concentrations, and activity were induced as well, along with GSH levels, in animals treated with atrazine for 3 and 4 days. However, treatment with atrazine over a longer period of time leads to variations in the expression of hepatic phase I CYP and phase II

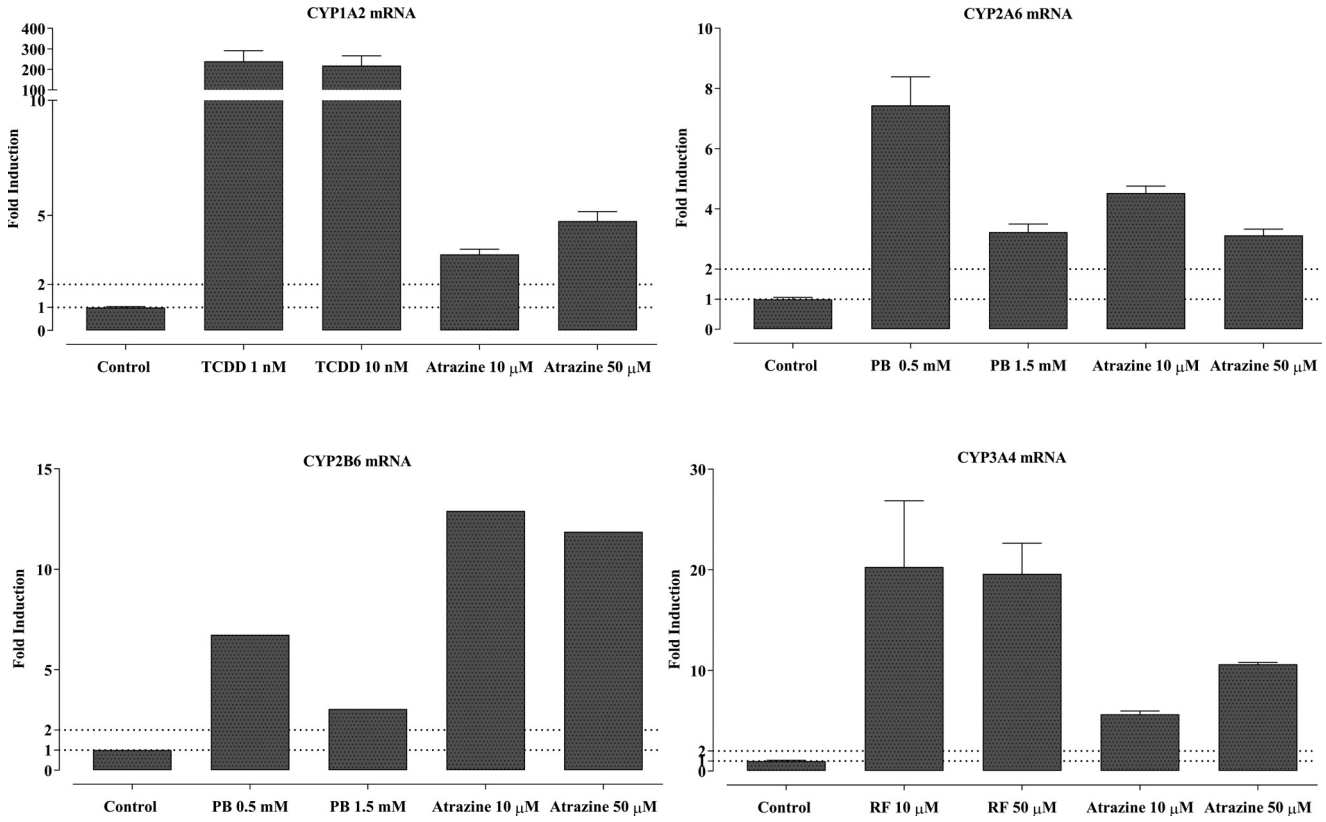


Fig. (2). CYP mRNA induction levels in the human hepatoma cell line HepaRG after incubation for 24 h with DMSO (control cells), atrazine, and model inducers: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (AHR agonist and CYP1A2 model inducer), phenobarbital (PB) (CAR activator and CYP2A6 and CYP2B6 inducer), rifampicin (RF) (PXR agonist and CYP3A4 inducer), or DMSO (control cells). CYP1A2, CYP2A6, CYP2B6 or CYP3A4 mRNA levels were measured by TaqMan probes using RT-PCR analysis. The mRNA levels were expressed relatively to those in control cells (considered as 1) and data are expressed as mean ± SD of six replicates (data from Abass *et al*. [36]). (*A higher resolution version of this figure is available in the electronic copy of the article*).

GST enzymes compared to shorter-term ATR treatment, which may be due to liver adaptation [38].

3.2. Metabolites of Potential Reactivity

There are two specific metabolite groups, which deserve further attention: carboxylated alkyl substituents of dechlorinateds-triazines and oxidized s-triazine amino groups, which have been linked to toxicities for some other chemicals. Carboxylic acid metabolites of dechlorinatedterbuthylazine in groundwater may be conjugated by UDP-glucuronosyl transferases (UGTs) to yield acyl glucuronides. These are linked to idiosyncratic drug toxicities and can be found in pharmaceuticals at relatively high (clinical) doses [39, 40]. Regarding triazines, kidney damage has been observed in several 90-day rat studies on terbuthylazine and other triazines, but kidney damage is possibly due to the physicochemical nature of triazine metabolites and their precipitation in the kidneys at high doses [41]. There have been no studies suggesting the potential role of acyl glucuronides in chloro-s-triazine toxicities. It is of interest that structurally exactly similar carboxylic acid metabolites of terbuthylazine have been observed also for cyanazine, another chloro-s-triazine herbicide [42]. These cyanazine metabolites did not cause toxicity at the highest dose used, approximately 1000 mg/kg bw/day, in the 90-day rat study.

Regarding N-oxidations, there are many reactions leading to reactive metabolites and intermediates resulting ultimately in tissue injuries, genotoxicity, and carcinogenicity [43, 44]. Although there are nitrogen-containing functional groups in all chloro-s-triazines, only one study has been published on N-oxidation and imine formation of the N-ethyl group of atrazine and subsequent formation of a glutathione conjugate [11]. However, on the basis of *in vitro* studies, it is practically impossible to suggest that glutathione conjugates predict reactive metabolite-based toxic reactions. This is because the nature and quantity of specific GSH-conjugates are dependent on the substance under study and on the specific *in vitro* systems used [39, 45]. Toxicological studies on atrazine or other triazines do not suggest potentially reactive metabolite-associated toxic outcomes. However, there are large gaps in knowledge.

3.3. Biological Activities

It has been observed that dechlorination decreases or abolishes herbicidal activities of chloro-s-triazines [46]. In mammalian systems, dechlorination is usually achieved by GSH conjugation, but in soil microbial systems, dechlorination is enhanced by the induction of specific microbial enzymes. The enzymes catalyze dechlorination of these herbicides so that after further metabolism and degradation, microbes are able to use an herbicide as a source of carbon and nitrogen [47].

Regarding mammalian toxicities, dechlorination has been demonstrated to change the toxicity spectrum of metabolites, and usually decrease the observed toxicity when compared with chlorine-preserving metabolic processes. For example, while atrazine has been shown to be an endocrine disruptor in the ED screening program's male and pubertal protocols, hydroxyatrazine exposure was devoid of such effects, although it caused kidney damage [48]. Also, in pubertal development in Wistar rats, hydroxyatrazine was less potent than chlorine-containing diamino-atrazine [49].

With respect to soil-produced groundwater metabolites, much less is known about their toxicities, but in those cases where experimental data are available, the importance of chlorine function seems similar with mammalian metabolites.

3.4. Toxicity Mechanisms

In regulatory toxicity studies of chloro-s-triazines, the most conspicuous findings are related to reproductive and developmental toxicity, especially disruptions of female developmental phases, which have been observed with practically all chloro-s-triazines. Further mechanistic studies indicate that chloro-s-triazines appear to have the endocrine mode of action (Table **4**) [50, 51]; the molecular site of action being in the central nervous system at the hypothalamus level, although also peripheral metabolism changes affect the response [52]. In a study on female Sprague Dawley (SD) rats administered atrazine in the diet for 6 months, the most sensitive effect was the suppression of the preovulatory luteinizing hormone (LH) surge [51], resulting in prolonged estrus in adult female rats, and developmental delays such as delayed vaginal opening and preputial separation in developing rats. The No Observable Effect Level (NOEL) was 1.8 mg/kg/day, which has been used by the USEPA [53] and WHO [54] to evaluate intermediate term and lifetime risks of exposure to atrazine and other chlorotriazines. Also, later regulatory assessments have concluded that the attenuated preovulatory surge is the most sensitive effect to be used in setting the NOEL value [5].

In the regulatory 2-year chronic toxicity and carcinogenicity studies with atrazine and simazine, mammary tumors were produced in female Sprague-Dawley rats. It is highly probable that these tumors are based on the above-described neuroendocrine mechanism of action and are species, strain and sex specific and most probably not operative in humans (see below human health outcomes section). Other common adversities, such as increased liver and kidney weight or decreased body weight gain, have been observed with all chloro-s-triazines. On the other hand, studies on teratogenicity, genotoxicity, and carcinogenicity have usually been negative, with the exception of the above-mentioned mammary tumors by atrazine and simazine.

Several other mechanisms for particular toxicities have been suggested, but they are interpreted to be less critical than the above-described neuroendocrine-associated toxicities. Suggestions on the role of pro-oxidant stress as a mechanism of toxicity of chlorotriazines have been presented [55]. However, this effect has been demonstrated either in *in vitro* experiments or at doses far higher than those causing neuroendocrine effects. Kidney toxicity of some metabolites is probably based on physicochemical properties, which are of importance in causing the precipitation of metabolites in kidney tubuli and consequent kidney damage [41].

Table 4. Endocrine disruptor effects of atrazine.

Species	Outcomes	References
Female rats	Activate the release of pituitary hormones at (75 mg/kg)	[56]
Female rats	Inhibit luteinizing hormone release from the pituitary (100-200 mg/kg)	[56, 57]
Female rats	Increase steroidogenic enzymes and sex steroid hormone levels (200 and 300 mg/kg)	[58, 59]
Female rats	Increase the estrogen-to-androgen ratio (300 mg/kg)	[58]
Female rats	With maximum tolerated dose, lengthening of the estrous cycle - Increased number of days in estrus or under the influence of exposure to estrogen - Earlier onset of galactocele formation - Earlier onset of mammary and pituitary tumor formation	[60]
Male and female rats	Affect reproductive functions	[50, 56]
Rats	Decrease serum and testicular testosterone levels (100-200 mg/kg body weight)	[61]
Rats	Inhibit luteinizing hormone (LH) and testosterone production (at or above 100 mg/kg per day)	[62]
Rats	Reduce serum and intratesticular testosterone levels at 50 mg/kg-bw/day	[63]
Oral exposure of peripubertal male rats	Down-regulate Leydig cell steroidogenesis, leading to inhibition of cAMP production and a severe decline in mRNA transcripts of several genes responsible for steroidogenesis	[64]
Male and female rats	Delay pubertal development	[65, 66]
Cultured rat pituitary and testicular Leydig cells	Act as a general endocrine disrupter by inhibiting cAMP-specific PDE4s	[67]
Primary rat granulosa cells	Increase progesterone and estradiol production and activity of aromatase at 10 μM	[68]
Swine granulosa cells	Disrupt steroidogenesis (0.1 and 10 μM)	[69]
Human adrenocortical carcinoma H295R cells	Elicit estrogen action by up-regulating aromatase activity	[70-73]
Human placental carcinoma cell line JEG-3	Up-regulate aromatase activity	[73]

Table 5. Selected MRLs for triazines.

Groups of Product	EFSA				Health Canada	
-	Atrazine	Simazine	Terbuthylazine	Cyanazine	Atrazine	Simazine
Default MRL mg/kg	-	-	-	0.01	-	-
Fruits, fresh or frozen; tree nuts	0.05	0.01-0.02	0.05-0.1	-	-	0.05**
Vegetables, fresh or frozen	0.05	0.01-0.02	0.05-0.1	-	-	-
Pulses	0.05	0.01	0.05-0.1	-	-	-
Oilseeds and oil fruits	0.05	0.01-0.05	0.05-0.1	-	-	-
Cereals	0.05	0.01	0.05-0.1	-	-	-
Teas, coffee, herbal infusions, cocoa and carobs	0.1	0.05	0.05*	-	-	-
Hops	0.1	0.05	0.1	-	-	-
Spices	0.1	0.05	0.05-0.1	-	-	-
Sugar plants	0.05	0.01	0.05	-	-	-
Products of animal origin -terrestrial animals	-	0.01	0.05	-	0.04*	-
Honey and other apiculture products	0.05	0.01	-	-	-	-

* Products of animal origin include fat, meat, and meat byproducts of cattle, goats, hogs, horses, poultry and sheep.
** For nuts only.
- Codex Alimentarius MRLs database: http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/pesticides/en/.
- Health Canada MRLs database. http://pr-rp.hc-sc.gc.ca/mrl-lrm/results-eng.php.
- EU Pesticide MRLs database: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN.

4. REGULATORY GUIDANCES AND PERMISSIBLE HUMAN EXPOSURES

4.1. Oral/Dietary Contaminant Intake Guidance Values

Table **5** presents a comparison of reference values for triazines. Oral or dietary guidance values are summarized as food consumption is the primary route of exposure to triazines. Estimates of exposure can be compared to reference or guidance values. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) defined it as the daily intake of a food additive, which, during the entire lifetime, appears to be without appreciable risk. The United States Environmental Protection Agency (USEPA) has offered an Acceptable Daily Intake (ADI) modification with the name Reference Dose (RfD) as the acceptable safety level for chronic non-carcinogenic and developmental effects. The Agency for Toxic Substances and Disease Registry (ATSDR) has established national values analogous to ADI, but named them as "Minimal Risk Levels" (MRLs). This abbreviation is identical to the Codex Alimentarius MRLs (Maximum Residue Levels), but the meaning is absolutely different; obviously this caused confusion in terminology that still exists today. ADIs, RfDs, and MRLs are usually based on studies carried out on laboratory animals but can be based on epidemiological studies.

4.2. Regulatory Limits in Food and Water (Food Safety Limits) for Chloro-s-triazines

According to the European Food Safety Authority (EFSA) the Maximum Residue Levels (MRLs) are defined as "the highest level of a pesticide that is legally tolerated in or on food or feed" [74]. Other agencies have similar definitions. MRLs for chloro-s-triazines in food and water are not a direct human health risk assessment parameter, but they do represent a level below which there is no concern for human health. Detailed MRL values have been set by EFSA for atrazine, simazine, and terbuthylazine, while limited numbers of MRLs were established by Health Canada for atrazine and simazine. So far, no MRL values in food for chloro-s-triazines have been published in the Codex Alimentarius (established by the Food and Agriculture Organization of the United Nations and the World Health Organization), (for a summary of MRL values, see Table **6**). Atrazine and its chloro-s-triazine metabolites - deethyl-atrazine, deisopropyl-atrazine and diaminochlorotriazine - have been found in surface water and groundwater consequent to the use of atrazine as a pre-emergent or early post-emergent herbicide. The metabolite hydroxyatrazine is more commonly detected in groundwater than in surface water. The guideline value for atrazine and its chloro-s-triazine metabolites in water is 0.1 mg/l (100 μg/l) and 0.2 mg/l (200 μg/l) for hydroxyatrazine [54, 75].

5. HUMAN BIOMONITORING

5.1. Human Exposure to Chloro-s-triazine Herbicides

Generally, there are many methods to assess human exposure. When it comes to collecting dietary information, food frequency questionnaires or recall surveys are used to collect information on possible sources of exposure. These can be combined with contaminant levels measured, or modeled, in food items to estimate exposure from food. Another estimate of total exposure is human biomonitoring data, or the measurement of a chemical (and its potential metabolites) in a biological matrix, such as blood or urine. However, studies on the impact of pollutants on human health are challenging to undertake because of many confounding factors influencing health at the same time.

Human biomonitoring (HMB) provides an estimate of exposure totriazines, with the "internal dose" resulting from integrated exposures from all routes. Tools are available to interpret HBM data in different contexts. There are three primary tools that are, in order of increasing complexity, reference values, biomonitoring equivalents, and tissue-based guidance values. However, no human guidance values on triazine herbicides are available, and the presence and profiles of triazine herbicides in human biological samples remain limited.

Table 6. Comparison of oral non-cancer MRLs, ADIs, and RfDs for chloro-s-triazines.

-	ATSDR	EFSA		US-EPA	Codex
Substance	Minimal Risk level (MRLs) mg/kg bw/day	ADI mg/kg bw/day	ARfD mg/kg bw	RfD mg/kg bw/day	ADI mg/kg bw/day
Atrazine	0.01 acute 0.003 Int.	0.02	0.1	0.035	-
Cyanazine	0.002	-	-	0.002	-
Propazine	-	-	-	0.02	-
Simazine	-	-	-	0.005	-
Terbuthylazine	-	0.004	0.008	-	-

- ATSDR. https://www.atsdr.cdc.gov/mrls/pdfs/ATSDR%20MRLs%20-%20May%202019-H.pdf.
- Codex Alimentarius database: http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/pesticides/en/.
- EU Pesticide MRLs database: http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN.
- US EPA Regional Screening Levels (RSLs). May 2019. Available online at: https://www.epa.gov/risk/regional-screening-levels-rsls-generic-tables.

Atrazine and simazine were quantified in human blood serum samples from the adult population in Jiangsu Province, China. The atrazine level was 0.05 ± 0.02 (Mean ± SD) ng/mL (n=5), while the simazine level was 0.83 ± 0.79 ng/mL (n= 170) [76]. Terbuthylazine was quantified in hair samples from farmers and rural residents, while its main metabolite in urine, desethylterbuthylazine, was detected in post-application samples of farmers from the province of Cremona, Lombardy, Italy [77, 78]. The median levels of terbuthylazine were 0.01 ng/mg hair in both the agriculture workers and rural residents before the application season, while levels were 0.08 ng/mg and 0.01 ng/mg, respectively, at the end of the application season. In the agriculture workers, the urinary median levels of desethylterbuthylazine were 0.0, 1.81, and 2.94 µg/L before, during, and after the application season, respectively [77, 78]. Another study from Lombardy estimated the exposure of 28 agricultural workers to terbuthylazine in real-life working conditions. Median daily exposure on the skin was 0.86 µmol (0.22-4.36) per worker. The estimated absorbed doses of terbuthylazine, based on the measured levels of skin and hand exposure and the percentage of dermal absorption, were 10-230 times below the Acceptable Operator Exposure Levels (AOEL) of 3.2 µg kg bw^{-1} day^{-1}, in terbuthylazine applicators. Terbuthylazine was not detected in all urine samples, while its main metabolite, N-t-butylammelide, was quantified only in 3 out of 16 post-exposure urine samples [79].

Although there are large uncertainties in estimating the real exposure *vis-a-vis* allowable exposures, *e.g.* ADIs, mainly due to inadequacy of appropriate data, it may be useful to perform some calculations. For example, on the basis of urinary desethylterbuthylazine levels [77], which were maximally about 3 µg/L, it is possible to estimate the daily internal exposure to terbuthylazine as 18 µg (assuming daily urine output 2 L and the desethyl metabolite 33% of the total terbuthylazine exposure). The ADI for a 70 kg subject is 280 µg and consequently the daily exposure of 18µg would be roughly 5% of the ADI under the above assumptions. Calculations based on Rubino *et al*. [79] data suggest maximal exposure of 22.4 µg, which is not too far from the value of 18 µg. In the future, physiologically-based toxicokinetic models should allow for more precise calculations when a more complete data based on human toxicokinetic characteristics are available.

5.2. Human Health Outcomes Associated with Chlorotriazine Herbicides

Epidemiological evidence of potential associations between chloro-s-triazine exposures and various cancers and reproductive toxicities have been reviewed recently [80-82], but conclusions remain rather uncertain (see below).

Atrazine has been the most researched chlorotriazinein human epidemiological studies. Systematic reviews of epidemiological evidence have been published on atrazine carcinogenicity [80, 81, 83-85], pregnancy outcomes [82], oxidative stress [86], and head and neck cancer [87].

On the basis of these systematic reviews, it has been concluded that the evidence of the link between atrazine exposure and the occurrence of cancer or birth defects in humans is too weak to make convincing conclusions. An early review of epidemiologic evidence concluded that, due to a lack of evidence and/or inadequate and limited published data, it is not possible to classify atrazine or chlorotriazines as carcinogenic in studied populations [83]. In 2011, Sathiakumar *et al*. [80] updated the earlier review. The main findings were the same; the conclusions from the 36 studies reviewed were limited by a lack of in-depth exposure measurements and by the small numbers of subjects with potential high exposure and/or with many years of follow-up since the first exposure. Therefore, the evaluated epidemiology studies did not provide consistent, scientifically convincing evidence of a causal relationship between exposure to atrazine or chlorotriazine herbicides and cancer in humans [80].

Similarly, based on a review of a large number of published epidemiologic studies conducted on pesticide applicators, workers, and farmer families, there was no causal association between triazines and cancer incidence in studied populations [85]. These findings were in line with a weight-of-evidence approach employed by the US EPA and the Scientific Advisory Panel to evaluate conflicting reports in the context of all the epidemiologic studies on the causal relationship between atrazine exposure and the occurrence of any specific cancer in humans. In essence, there was no causal association between atrazine and cancer, and occasional positive findings can be attributed to bias or chance [81]. Similar conclusions were made on the causal link between atrazine exposure and birth defects in humans. The poor quality of the data and the lack of robust findings across published epidemiologic studies prevented confirmation of the link [82].

On the other hand, risks of prostate cancer were observed among individuals exposed to organochlorine pesticidesin California's intensely agricultural Central Valley, but not among those exposed to a simazine herbicide [88]. Mills and Yang [89] investigated breast cancer incidence rates using negative binomial regression models of pesticide usage data among California Hispanic

females, who are commonly employed in agricultural operations. Breast cancer was not significantly associated with pounds of atrazine and simazine. Moreover, there were no apparent associations between simazine levels in house dust and risk of childhood acute lymphoblastic leukemia in California [90]. Inadequate evidence humbled the associations between exposure to atrazine or simazine and a higher risk of breast, prostate, or ovarian cancer, non-Hodgkin's lymphoma, or any other cancer type in humans [85].

Inhalation risks to California communities from airborne agricultural pesticides, including simazine, were assessed by probability distribution analysis using ambient air data [91]. Simazine exposure estimates were way below non-cancer reference values of the exposed populations (adults and children). Lifetime cancer risks from exposure to simazine were below regulatory scrutiny of cancer risk (1×10^{-6}) being 2, 3, and 5 x 10^{-8} for the 50^{th}, 75^{th}, and 95^{th} percentile probability estimates, respectively [91]. Prostate cancer risk in California farm workers from a nested case-control study of prostate cancer within a large cohort of a predominantly Hispanic labor union was evaluated [92]. The study concluded that Hispanic farm workers with relatively high levels of exposure to simazine experienced elevated risk of prostate cancer compared to workers with lower levels of exposure. Additionally, a significant association (crude odds ratio (OR) = 1.89; 95% CI: 1.08-3.33 for high exposure) between prostate cancer risk and exposure to simazine in British Columbia farmers was reported [93].

There are some evidence suggesting that exposure to herbicides, including atrazine and simazine, is associated with an increased risk of Parkinson disease (PD) in prevalent cases of PD from Colorado [94] as well as allergic and non-allergic wheeze among male farmers in North Carolina and Iowa [95].

Cyanazine carcinogenicity was evaluated among exposed licensed pesticide applicators (n= 57, 311) who live in Iowa and North Carolina through the Agricultural Health Study (AHS). Due to limited numbers for certain cancers, the study did not provide any clear, consistent associations between cyanazine exposure and any cancer analyzed [96].

Results from the same AHS prospective study showed that cyanazine was associated (OR = 1.88, 95% CI = 1.00, 3.54), with chronic bronchitis among nonsmoking farm women (n = 21, 541) [97]. In addition, the odds of diabetes incidence increased with both cyanazine ever use and cumulative days of use among male licensed pesticide applicators enrolled in the AHS [98]. Moreover, parental exposure to cyanazine during the 3 months prior to conception was associated (odds ratio = 4.99, 95% confidence interval: 1.63-15.27, n=3412 pregnancies) with increased risk of birth defects in male offspring from the Ontario Farm Family Health Study. However, the authors stressed that further investigations are required to verify the findings [99].

CONCLUSION

Chloro-s-triazines are a rather homogeneous group of herbicides with apparently similar mechanism of action, adversities in regulatory animal toxicology studies, and general toxicokinetic behavior. Interestingly, based on apparent similarities it is difficult to pinpoint any specific toxicokinetic or toxicological features to explain why atrazine is the predominant herbicide in the USA, while it is not marketed in the EU. In the EU, terbuthylazine is the major herbicide of this group. However, the general similarity may prove to be deceptive at a closer look because of two reasons: the scientific data base is rather patchy and regulatorily biased, *i.e.* gaps in knowledge abound, and a few in-depth, mainly *in vitro,* studies point to important differences between individual compounds.

It is obvious on the basis of the above survey that integration of toxicokinetic data and animal toxicodynamic observations with human *in vivo* and epidemiological data will become possible only after a concerted effort to produce appropriate and more precise information. In this respect, work along the lines of Integrated Approaches to Testing and Assessment (*IATA*) is a promising way forward; here, chemical safety assessment is based on the integration and translation of the data derived from multiple methods and sources with the possibility of building a tentative network of adverse outcome pathways [100].

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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