

2. Joint Toxic Action Data for the Mixture of Concern and Component Mixtures

This chapter provides a review and evaluation of the literature pertinent to joint toxic action of the mixture and its components.

2.1 Mixture of Concern

Toxicological data or PBPK models were not available for the complete mixture of concern.

2.2 Component Mixtures

Toxicological and mechanistic data, but no PBPK models, were available for some of the binary mixtures. With the exception of the data for the joint action of the triazines, these data were fairly limited. Atrazine and deethylatrazine are generally considered together as one component in this profile, because of the similarity in their metabolism and mechanism of action, and because deethylatrazine is a metabolite and environmental degradation product of atrazine (Appendix A).

2.2.1 Atrazine/Deethylatrazine and Simazine

In a study of neuroendocrine/reproductive effects in mature male Atlantic salmon parr (salmon living in fresh water), short-term exposure of the olfactory epithelium (*in situ*) to atrazine (1.0 µg/L) or simazine (1.0 or 2.0 µg/L) significantly reduced the olfactory response to the female priming pheromone, prostaglandin F_{2α} (Moore and Lower 2001). The response was determined electrophysiologically in anesthetized fish. Exposure to a mixture of the two herbicides as a 1:1 mixture at total concentrations of 1.0 and 2.0 µg/L resulted in reductions that were not significantly different from the single chemicals at the same concentrations. Thus, results indicated concentration (dose) addition. Similar experiments with the individual chemicals and mixtures studied the impact on the reproductive priming effect of prostaglandin F_{2α} on the levels of expressible milt and on plasma levels of testosterone, 11-ketotestosterone, and 17,20β-dihydroxy-4-pregnen-3-one in unanesthetized fish exposed for 5 days. Results indicated additivity with regard to a reduction in expressible milt and on hormonal status.

Atrazine and simazine were tested for concentration addition in green algae, using the inhibition of reproduction of synchronized cultures of *Chlorella fusca* during one generation as the endpoint (Faust et al. 1993). The observed median effective concentration (EC₅₀) of the mixture was virtually the same

as that predicted on the basis of concentration addition. This result was expected because both herbicides inhibit photosystem II. The experimental design was adequate to support this conclusion, but the relevance to human health is questionable.

A study in Chinese hamster ovary (CHO) cells incubated with atrazine and/or simazine used the coefficient of variation of the G1 peaks (nuclei) and of the largest chromosome peak (isolated chromosomes) as indices of clastogenicity (Taets et al. 1998). Chromosome breakage causes uneven distribution of DNA in nuclei of daughter cells, increasing the variability in nuclear size and hence the coefficient of variation; a similar impact is seen on chromosome size and variability. When tested at the levels of EPA MCLs (0.003 mg/L atrazine, 0.001 mg/L simazine, or mixture of 0.003 mg atrazine plus 0.001 mg/L simazine), the coefficient of variation for the G1 peaks was significantly elevated to a similar extent for both herbicides individually and for the mixture as compared with controls. Similar results were seen for the coefficient of variation for the largest chromosome peak, but the increase for simazine alone was not statistically significant. When tested at the highest levels found in Illinois water supplies (0.018 mg/L atrazine, 0.004 mg/L simazine, or mixture of 0.018 mg/L atrazine plus 0.004 mg/L simazine), similar results were found for G1 peaks. For the largest chromosome peak, however, results are uncertain because the description of the results for atrazine in the text, the table, and the figure are not consistent. Limitations of this study include lack of statistical comparison of results from the mixtures with the single chemicals, higher combined dose of chemicals in the mixture than in the single chemical treatments, and the inconsistent reporting of results for atrazine. Under dose addition, a higher degree of clastogenicity would be expected from the mixtures as compared with the single chemicals in this study, but the higher combined dose in the mixture groups may have been more cytotoxic. Cytotoxicity, according to the study authors, would tend to result in selection for resistant cell types that are more homogeneous, which would lower the coefficient of variation. Thus, the study design and results are inadequate to support meaningful conclusions regarding the type of joint action.

Neither atrazine nor simazine nor the mixture of the two produced a mutagenic response in *Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, or TA100 with or without a rat liver S-9 activating system (Eisenbeis et al. 1981). A range of concentrations was tested from 'full strength' down to zero; details were not provided.

Analysis of studies of mode of action of certain triazine herbicides, including atrazine and simazine, and their chlorinated metabolites, including deethylatrazine, has indicated that they have a common mechanism of toxicity with regard to attenuation of the luteinizing hormone (LH) surge in female and

male rats, alteration of the estrous cycle, delayed pubertal development in both sexes of rats, and altered pregnancy maintenance (EPA 2002c). These triazines are not estrogenic. Rather, their mechanism of reproductive toxicity involves neuroendocrine disruption of hypothalamic-pituitary-gonadal function. In female Sprague-Dawley rats, atrazine accelerates the normal process of reproductive senescence, which is initiated by a failure of the hypothalamus to release levels of gonadotropin releasing hormone (GnRH) that are adequate to stimulate the pituitary to release LH. Without sufficient LH, ovulation does not occur, estrogen levels remain high, persistent estrus occurs, and mammary gland tumors develop. In other strains of rats, atrazine causes elevated progesterone levels, which leads to pseudopregnancy and persistent diestrus, but not mammary tumors. The carcinogenic outcome is not expected in humans, due to species and strain differences in reproductive senescence. Reproductive senescence in female Sprague-Dawley rats involves decreasing hypothalamic function and *increased* serum estrogen levels (thought to contribute to mammary gland cancer), whereas reproductive senescence in women involves ovarian depletion and *decreased* serum estrogen levels (ATSDR 2003; EPA 2002a, 2002b, 2002c). Further mechanistic detail is provided in Section A.3 of Appendix A. Although the carcinogenicity of these triazines in female Sprague-Dawley rats is not thought to be applicable to humans, the neuroendocrine disruption at the level of the hypothalamus, resulting in altered hypothalamic-pituitary function, is considered to be relevant to humans. The mode of action of atrazine, deethylatrazine, and simazine with regard to reproductive function and reproductive development is expected to be dose additive (EPA 2002c).

2.2.2 Atrazine and Diazinon

No studies of this binary mixture in mammals were located. A study on the joint toxic action of diazinon and atrazine in midge (*Chironomus tentans*) larvae reported that environmentally relevant concentrations of atrazine (40–200 µg/L) potentiated the acute neurotoxicity of diazinon (7.7–29.7 µg/L) in 96-hour static toxicity tests (Belden and Lydy 2000). Acute neurotoxicity was measured as the inability of the midges to perform normal swimming motions. Based on changes in the EC50 values, atrazine treatment at 40, 80, and 200 µg/L increased the toxicity of diazinon 1.81-, 2.11-, and 2.71-fold, respectively. No effect on the diazinon EC50 was seen at 10 µg/L atrazine. Atrazine alone was not acutely toxic to midges even at the limit of water solubility (10,000 µg/L), which was 50 times the highest atrazine concentration used in the study. The effect of atrazine on diazinon toxicity may have been mediated through induction of cytochrome P450 enzymes that activate organophosphorus insecticides. This conclusion is based on induction of the metabolism and potentiation of the neurotoxicity of another organophosphorus insecticide, chlorpyrifos, by atrazine in additional experiments in this study.

Additional studies of joint toxic action of atrazine and diazinon in an aquatic amphipod (*Hyalella azteca*, a small shrimp-like creature) also reported potentiation of diazinon toxicity by atrazine (Anderson and Lydy 2002). In 96-hour static toxicity (median lethal concentration [LC50]) assays, atrazine at 80 and 200 µg/L increased the acute toxicity of diazinon 2.0- and 3.0-fold, respectively. No effect on the diazinon LC50 was seen at ≤40 µg/L atrazine. Atrazine alone was not lethal at a concentration of 10,000 µg/L. In additional studies on acetylcholinesterase activity, atrazine alone (200 µg/L) had no effect during 96-hour static tests. At the 96-hour LC01 for diazinon (0.90 µg/L), in comparison with controls, acetylcholinesterase activity was 27% lower with diazinon alone, and 43% lower with diazinon and atrazine (200 µg/L), indicating a potentiation by atrazine of diazinon toxicity.

In the common house fly (*Musca domestica*), however, atrazine (200 or 2,000 ng/mg body weight = 0.2 or 2 µg/mg body weight) did not affect the acute lethality of diazinon (0.2–9.9 ng/mg body weight), when both chemicals were applied in acetone to the ventral abdomen (Anderson and Lydy 2002). To test whether the atrazine penetrated the cuticle, a much smaller dose of radiolabeled atrazine (1.27 ng/mg body weight) was applied to the ventral abdomen in the same volume of acetone as for the joint action study; radioactivity did appear to penetrate the cuticle. This experiment did not demonstrate whether or not atrazine was actually absorbed by the flies. The studies in midge larvae and in amphipods indicate that potentiation may not be seen at low doses of atrazine. Thus, it is unclear whether the lack of potentiation by atrazine in flies represents a species difference, or whether the atrazine dose actually absorbed by the flies was too low to be effective. Oral administration of atrazine may result in a higher internal dose, but was not tested.

Diazinon is metabolically activated by cytochrome P450 to diazoxon, which binds to acetylcholinesterase, inhibiting the ability of this enzyme to hydrolyze acetylcholine, a neurotransmitter. This inhibition results in continued neurological stimulation. Acetylcholinesterase inhibition is the principal toxic effect in humans and animals, including insects. Thus, the results in the studies in midges and amphipods may be applicable to humans, and indicate greater-than-additive influence of atrazine on diazinon neurotoxicity.

2.2.3 Simazine and Diazinon

No studies of this binary mixture were located. Data from the atrazine-diazinon mixture, reviewed in the previous section, may be relevant because of the similarities between simazine and atrazine. Reasoning

by analogy with atrazine, the influence of simazine on diazinon neurotoxicity would be expected to be greater than additive.

2.2.4 Atrazine and Nitrate

The potential for a chemical interaction between atrazine and nitrite (the metabolite of nitrate) resulting in the formation of N-nitrosoatrazine has been investigated. The formation of N-nitrosamines from pesticide amino groups and nitrite is of concern because most N-nitrosamines are carcinogenic (Lijinsky 2001; Preussmann and Stewart 1984).

Atrazine and nitrite have been shown to react at acidic pH to form N-nitrosoatrazine (Eisenbrand et al. 1975b; Krull et al. 1980; Mirvish et al. 1991; Wolfe et al. 1976). N-Nitrosoatrazine has been tentatively identified in Mississippi River water and New Orleans drinking water (Fine et al. 1976). No formation of N-nitrosoatrazine was detected in soils adjusted to pHs of 2.5–5.5 and incubated with atrazine and a molar excess of nitrate (limit of detection 10 ppb) for 1–3 months (Kearney et al. 1977). Similar incubation with nitrite, however, resulted in the formation of a small amount of N-nitrosoatrazine at 1 week at pHs of 2.5–5.3, but no nitrosoatrazine was detected at 4 or 10 weeks. Thus, it is unclear whether or not N-nitrosoatrazine could result from nitrate and atrazine in soil, as the initial measurements in that experiment were made after 1 month of incubation. Additional experiments in which N-nitrosoatrazine was added to soil showed that the nitrosamine was degraded (denitrosated to atrazine) (Kearney et al. 1977). N-Nitrosoatrazine was stable in water at 25 °C at pHs above 4 in the dark, but was rapidly decomposed to atrazine and deethylatrazine by light (Wolfe et al. 1976).

The formation of N-nitrosoatrazine from atrazine and nitrite has been demonstrated in human gastric juice (pH 1.5–2.0) during 1.5–12 hours of incubation at 37 °C (Cova et al. 1996). The percent formation peaked at 3 hours, and gradually declined thereafter, due to degradation of N-nitrosoatrazine to atrazine. Peak formation of N-nitrosoatrazine was 2% from 0.05 mM atrazine and 0.5 mM nitrite, 23% from 0.05 mM atrazine and 3 mM nitrite, and 53% from 1 mM atrazine and 3 mM nitrite. The levels of nitrite used were similar to peak gastric levels of nitrite (1.77 mM) in subjects who ingested a salad-type meal containing 1.15 mM of nitrate (Walters et al. 1979).

The formation of N-nitrosoatrazine from atrazine and nitrite also has been demonstrated *in vivo*. Approximately 0.04% conversion occurred within 15 minutes in mice gavaged with 1,000 µg atrazine followed by 500 µg nitrite (Krull et al. 1980). At 500 µg atrazine and 500 µg nitrite, N-nitrosoatrazine

was found in some but not all of the mice, and at 250 µg atrazine and 500 µg nitrite, N-nitrosoatrazine was not detected. The *in vitro* studies conducted as part of this study resulted in conversion of about 0.4% of the atrazine to N-nitrosoatrazine during incubation of 500 µg atrazine with 500 µg nitrate at 37 °C and pH 3 for 2 hours. According to Seiler (1977), the pH of the mouse stomach is approximately 4–5.

A study of cancer rates and drinking water contamination with atrazine (50–649 ng/L) and nitrate (0–91 mg/L) in Ontario “agroecosystems” reported that stomach cancer incidence was positively associated with atrazine concentrations and negatively associated with nitrate concentrations in drinking water (Van Leeuwen et al. 1999). Atrazine concentrations were negatively associated with colon cancer incidence. Associations with other cancer types were not observed. Atrazine and nitrate concentrations in drinking water were positively correlated. The analyses controlled for potential confounding factors such as age and smoking. Limitations of the study include the collection and analysis of data for ecodistricts rather than individuals. In addition, the exposure data were from the same time period as the cancer incidence data. The development of cancer, however, usually involves a latency period, such that previous exposure levels may be more important than concurrent exposure levels. This study does not establish causality, and is not supported by other studies of atrazine or nitrate (see Appendices A and D). Because no cancer type was positively correlated with both atrazine and nitrate concentrations, the study does not provide suggestive evidence of a greater-than-additive interaction as might be expected from nitrosamine formation, but interpretation of the study findings, and particularly the negative correlations, is problematic due to the limitations discussed previously.

The joint toxic action of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae was tested (Allran and Karasov 2000). Three concentrations of atrazine (0, 20, and 200 µg/L) and three of nitrate (0, 5, and 20 mg NO₃-N/L) were tested in a factorial design for a total of nine treatments. The selected concentrations bracketed the environmentally relevant range. Neither atrazine nor nitrate nor the mixtures had a significant effect on development rate, growth rate, percent metamorphosis, time to metamorphosis, percent survival, mass at metamorphosis, or hematocrit. Although these results suggest that environmental levels of atrazine and nitrate do not affect the development of the frog, they do not provide useful information on the mode of joint toxic action because the treatments were without effect on the endpoints studied.

The newt (larvae) micronucleus assay gave no indication of clastogenicity for atrazine alone, atrazine with nitrate or nitrite, nitrate alone, and nitrite alone (L’Haridon et al. 1993). Atrazine, nitrate, and nitrite

were tested at levels found in contaminated surface waters, plus nitrate and nitrite were tested at much higher levels. Some of the experiments included preincubation of the mixtures in the dark, to allow for chemical interaction, while minimizing the photodegradation of any N-nitrosoatrazine that might be formed. Testing of N-nitrosoatrazine itself gave slight but statistically significant positive results for clastogenicity at the two highest concentrations. These concentrations (7.5 and 15 ppm, corresponding to 30.6 and 61.2 μM), however, were much higher than could be generated from the concentration of atrazine tested (0.3 ppm, 1.4 μM), even if 100% of the atrazine was converted to the nitrosamine. Thus, if N-nitrosoatrazine is formed from atrazine and nitrate or nitrite in a polluted aquatic environment, the amounts formed may be too low to cause clastogenicity in newt larvae. This study raises concerns for the genotoxicity of N-nitrosoatrazine, but does not provide direct information regarding the mode of joint toxic action of atrazine and nitrate.

Another joint toxic action study used concentrations of atrazine, nitrate, and nitrite alone that were nonclastogenic in human lymphocytes *in vitro*, and half of these non-effective concentrations for testing of the binary mixtures of atrazine and nitrate or atrazine and nitrite (Meisner et al. 1993). No clastogenicity was seen for the mixtures. The concentration of atrazine (0.005 mg/L) in the binary mixtures, while too low to be effective itself, was 5-fold higher than a clastogenic concentration of N-nitrosoatrazine in another experiment reported in the same paper. The yield of N-nitrosoatrazine (if any) under these culture conditions, however, is not known.

A few studies have compared the genotoxicity of atrazine with that of N-nitrosoatrazine. Although these studies did not include investigations of the joint toxic action of atrazine and nitrate or nitrite, they are relevant to the issue of whether the chemical interaction of atrazine and nitrite results in a more toxic chemical, and therefore could be regarded as greater than additive. These studies are summarized below.

Neither atrazine nor N-nitrosoatrazine was mutagenic in *S. typhimurium* TA98, TA100, or TA1537 with or without rat liver S9 (Ishidate 1983; Ishidate et al. 1981), but N-nitrosamines are known to be more readily activated to bacterial mutagens by hamster liver S9 than by rat liver S9 (Lijinsky 2001). Human liver S9 also may be more active than rat liver S9 in N-nitrosamine activation, based on results with a single compound, dimethylnitrosamine (Hakura et al. 2003).

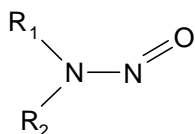
N-nitrosoatrazine was clastogenic in cultured human lymphocytes at concentrations 10,000 times lower than required for atrazine clastogenicity and 1,000 times lower than required for nitrate clastogenicity in the same assay (Meisner et al. 1993). In addition, N-nitrosoatrazine was mitogenic, whereas atrazine and

nitrate were not. In a Chinese hamster cell line derived from lung fibroblasts, N-nitrosoatrazine caused chromosomal aberrations when tested at a concentration 17-fold lower than an atrazine concentration (250 mg/L) that did not cause chromosomal aberrations in the same study (Ishidate 1983; Ishidate et al. 1981). Results of these studies indicate that N-nitrosoatrazine is more clastogenic than atrazine or nitrate, and stimulates cell division whereas atrazine and nitrate do not. This raises a concern that the formation of N-nitrosoatrazine through chemical interaction may be a greater-than-additive interaction in terms of genotoxic and proliferative effects. Implications for carcinogenicity or other effects are less clear.

The metabolism of N-nitrosoatrazine was compared with that of atrazine after oral administration of 50 mg/kg of either chemical to the rat (Meli et al. 1992). The cumulative percentage of the dose of atrazine excreted in the urine as atrazine and metabolites by 96 hours was approximately 37%, whereas for N-nitrosoatrazine, it was approximately 2%. Very little unchanged atrazine and no unchanged N-nitrosoatrazine were detected in urine. The primary urinary metabolite for both compounds was diaminochlorotriazine. *In vitro* studies of the metabolism of atrazine and N-nitrosoatrazine (2 mM of each) in 30-minute incubations with hepatic S9 fractions from untreated rats showed that 37% of the atrazine was metabolized versus 32% of the N-nitrosoatrazine. The total recovery of atrazine plus metabolites was 82%, whereas the total recovery of N-nitrosoatrazine and metabolites was only 39%. For atrazine, 44% of the recovered material was parent compound, whereas for N-nitrosoatrazine, only 7.4% was parent compound. A possible explanation for the low recovery of N-nitrosoatrazine and metabolites *in vivo* and *in vitro* is that N-nitrosoatrazine may be metabolized to reactive intermediates that bind to constituents of the body or the S9 fraction.

No full report of the joint toxic action of atrazine and nitrate or nitrite on carcinogenic endpoints or of the carcinogenicity of N-nitrosoatrazine has been published in the peer-reviewed literature. The class of compounds to which N-nitrosoatrazine belongs, the N-nitrosamines, has been extensively studied, particularly with regard to carcinogenicity.

The N-nitrosamines have the general structure:



in which R₁ and R₂ are alkyl or aryl moieties, and may have functional groups. Depending on whether R₁ and R₂ are the same, different, or joined, the nitrosamine is called symmetrical, asymmetrical, or cyclic. N-Nitrosamines have been extensively studied. Preussmann and Stewart (1984) reported that 86% of the

232 N-nitrosamines that had been tested for carcinogenicity in animals gave positive results. Many of the remaining 14% had been tested at below the maximum tolerated dose and/or in only one species, so the apparent negative results were not definitive. A few of the tested nitrosamines were unsymmetrical alkylarylnitrosamines, as is N-nitrosoatrazine (and N-nitrososimazine) (see Appendix E for structures); some of these alkylarylnitrosamines gave positive results for carcinogenicity. In addition, “high” (relative to expected human exposures) doses of amine compounds and nitrite administered in water and/or food to rats and mice induced tumors of the same type and at the same sites as expected for the corresponding nitrosamine. Tumors did not result from the amine compound or nitrite alone. Few data are available regarding the genotoxicity of N-nitrosamines in mammalian cells, so correlations between mammalian genotoxicity and carcinogenicity cannot be established (Lijinsky 2001; Preussmann and Stewart 1984). Therefore, the potential for human cancer risk is still unresolved and further studies are needed.

2.2.5 Simazine and Nitrate

The potential for a chemical interaction between simazine and nitrite (the metabolite of nitrate) to form N-nitrososimazine has been investigated. As mentioned previously for atrazine and nitrate, the formation of N-nitrosamines from pesticide amino groups and nitrite is of concern because most N-nitrosamines are carcinogenic (Lijinsky 2001; Preussmann and Stewart 1984). Simazine and nitrite were shown to react at acidic pH to form N-nitrososimazine (Eisenbrand et al. 1975b).

An *in vivo* study also suggests the formation of N-nitrososimazine from simazine and nitrite (Dmitrenko et al. 1996). Gavage administration of radiolabeled simazine at 2.3 mg/kg and sodium nitrite at 20.5 mg/kg resulted in an increase in labeled N-nitrososimazine in the liver and thymus relative to amounts formed from simazine alone at the same dose as in the mixture. Levels in kidney and spleen appeared elevated from coadministration of simazine and nitrite, but were not statistically significantly different from those obtained with simazine alone. No other tissues were analyzed. The levels of N-nitrososimazine formed in the absence of administered nitrite were attributed to endogenously formed nitrites and nitrogen oxide.

Neither simazine nor N-nitrososimazine was mutagenic in *S. typhimurium* TA98, TA100, or TA1537 with or without rat liver S9 (Ishidate 1983), but N-nitrosamines are known to be more readily activated to bacterial mutagens by hamster liver S9 than by rat liver S9 (Lijinsky 2001). Human liver S9 also may be

more active than rat liver S9 in N-nitrosamine activation, based on results with a single compound, dimethylnitrosamine (Hakura et al. 2003).

In a comparison of the potential clastogenicity of simazine and N-nitrososimazine in a Chinese hamster cell line originally established from lung fibroblasts, N-nitrososimazine produced chromosomal aberrations at 15 mg/L, but simazine did not at a 3-fold higher concentration (45 mg/L) (Ishidate 1983). This result raises the concern that the chemical interaction between simazine and nitrite may be greater than additive because it results in a new chemical that is more genotoxic than simazine. Implications for carcinogenicity or other effects are less clear.

No report of the joint toxic action of simazine and nitrate or nitrite on carcinogenic endpoints or of the carcinogenicity of N-nitrososimazine has been published. The N-nitrosamine class of chemicals, to which N-nitrosoatrazine belongs, has been studied extensively with regard to carcinogenicity. A discussion on this subject and its relevance to the potential carcinogenicity of N-nitrosoatrazine and N-nitrososimazine is provided at the end of Section 2.2.4.

2.2.6 Diazinon and Nitrate

No studies of the joint action of diazinon and nitrate were located.

2.3 Relevance of the Joint Toxic Action Data and Approaches to Public Health

This mixture was chosen because of its occurrence in rural well water. Thus, the expected exposures are intermediate to chronic oral exposures. No epidemiological or toxicological studies of the complete mixture are available. No PBPK models are available for the complete mixture or for any of the submixtures. Some information and studies are available for binary mixtures of the components, but they are not adequate to support a quantitative assessment of interactions. Therefore, the WOE approach is appropriate (ATSDR 2001a, 2001b) to predict the potential impact of interactions.

The binary weight-of-evidence (BINWOE) classification scheme is summarized in Figure 1. Rationales for the BINWOE determinations are presented in Tables 1–5 at the end of this section. The endpoints of particular interest for BINWOE determination were reproductive (atrazine, its metabolite deethylatrazine, and simazine), neurological (diazinon), hematological (nitrate), and carcinogenic (triazine reaction with nitrite to form N-nitrosamines). Insufficient information was available, however, for some of these

endpoints and binary mixtures, resulting in classifications of “indeterminate,” which are not presented in the tables in this section.

The BINWOE determinations are presented for the binary mixtures in the same order as these mixtures were considered in Section 2.2. Atrazine and deethylatrazine were generally considered together as one component, because of the similarity in their metabolism and mechanism of action, and because deethylatrazine is a metabolite and environmental degradation product of atrazine (Appendix A).

The predicted directions of interaction were additive with high confidence for atrazine/deethylatrazine and simazine on reproductive toxicity, greater-than-additive with medium confidence for the effects of atrazine/deethylatrazine or simazine on the neurotoxicity of diazinon, and greater-than-additive with low confidence for the joint toxic action of atrazine or simazine with nitrate on carcinogenicity. BINWOEs were indeterminate for the effect of diazinon on atrazine/deethylatrazine, simazine, and nitrate, and for the effect of nitrate on diazinon.

Figure 1. Binary Weight-of-Evidence Scheme for the Assessment of Chemical Interactions*

Classification	
Direction of Interaction	
=	Additive
>	Greater than additive
<	Less than additive
?	Indeterminate
Quality of the Data	
Mechanistic Understanding	
I.	Direct and Unambiguous Mechanistic Data: The mechanism(s) by which the interactions could occur has been well characterized and leads to an unambiguous interpretation of the direction of the interaction.
II.	Mechanistic Data on Related Compounds: The mechanism(s) by which the interactions could occur has not been well characterized for the chemicals of concern but structure-activity relationships, either quantitative or informal, can be used to infer the likely mechanisms(s) and the direction of the interaction.
III.	Inadequate or Ambiguous Mechanistic Data: The mechanism(s) by which the interactions could occur has not been well characterized or information on the mechanism(s) does not clearly indicate the direction that the interaction will have.
Toxicological Significance	
A.	The toxicological significance of the interaction has been directly demonstrated.
B.	The toxicological significance of the interaction can be inferred or has been demonstrated for related chemicals.
C.	The toxicological significance of the interaction is unclear.
Modifiers	
1.	Anticipated exposure duration and sequence.
2.	Different exposure duration or sequence.
a.	<i>In vivo</i> data
b.	<i>In vitro</i> data
I.	Anticipated route of exposure
ii.	Different route of exposure

* Adapted from: ATSDR 2001a, 2001b

Table 1. Effect of **Atrazine/Deethylatrazine** on **Simazine**: Reproductive Toxicity
 Effect of **Simazine** on **Atrazine/Deethylatrazine**: Reproductive Toxicity

BINWOE: =IA

Direction of Interaction - The direction of interaction is expected to be additive, based on a common mechanism of toxicity with regard to reproductive effects, similar metabolic fate, and additive joint toxic action on reproductive endpoints in the salmon (Moore and Lower 2001).

Mechanistic Understanding - Analysis of studies of mode of action of certain triazine herbicides, including atrazine and simazine, and their chlorinated metabolites, including deethylatrazine (metabolite of atrazine) and diaminochlorotriazine (metabolite of atrazine, deethylatrazine, and simazine), have indicated that they have a common mechanism of toxicity with regard to attenuation of the LH surge in female and male rats, alteration of the estrous cycle, delayed pubertal development in both sexes of rats, and altered pregnancy maintenance (EPA 2002c). The mechanism involves neuroendocrine disruption of hypothalamic-pituitary-gonadal function. The neuroendocrine disruption is expected to be relevant to humans. The mode of action of atrazine, deethylatrazine, and simazine with regard to these effects on reproductive function and reproductive development is expected to be dose additive. The appropriate rating for mechanistic understanding is I.

Toxicological Significance - Results of a study of the effect of atrazine and simazine on neuroendocrine and reproductive effects in mature male Atlantic salmon parr indicated concentration addition for reduced olfactory response, reduced levels of expressible milt, and on hormonal status in response to the female priming pheromone, prostaglandin F_{2α} (Moore and Lower 2001). Other studies of joint toxic action showed no mutagenic effects of either chemical or the mixture on *S. typhimurium* (Eisenbeis et al. 1981), and concentration addition with regard to inhibition of reproduction of cultures of *C. fusca* (green algae) (Faust et al. 1993). The toxicological relevance of the result in algae, which reflects inhibition of photosystem II, to humans is questionable. The mutagenicity study does not raise concerns for greater-than-additive toxicity. The neuroendocrine and reproductive effects in salmon have toxicological significance to the effects of concern for humans, despite the species difference. In mammals, the similarity in the reproductive effects of these chemicals together with the mechanistic understanding that indicates a common mechanism of toxicity for atrazine, deethylatrazine, and simazine strongly support the prediction of additivity. Therefore, a rating of A is chosen for toxicological significance.

Table 2. Effect of **Atrazine/Deethylatrazine** on **Diazinon**: Neurological Toxicity**BINWOE: >IIB**

Direction of Interaction - The direction of interaction is expected to be greater-than-additive, based on the potentiation of diazinon neurotoxicity by atrazine in the midge (Belden and Lydy 2000), potentiation of diazinon lethality and acetylcholinesterase inhibition in amphipods (Anderson and Lydy 2002), and induction by atrazine of metabolic activation of a similar organophosphorus insecticide (Belden and Lydy 2000), and similar mechanism of neurotoxicity in invertebrates and humans.

Mechanistic Understanding - Diazinon is a phosphorothioate organophosphorus insecticide that is metabolically activated through oxidative desulfuration to diazoxon by cytochrome P450. Diazoxon binds to acetylcholinesterase, inhibiting its ability to hydrolyze the neurotransmitter acetylcholine. The resulting accumulation of acetylcholine at the nerve endings causes continual neurological stimulation. This mechanism of action applies to both invertebrates and mammals. Atrazine induced the metabolic activation of a similar phosphorothioate organophosphorus insecticide, chlorpyrifos, and potentiated its acute neurotoxicity to midges (Belden and Lydy 2000). Based on the similarity in structure and mechanism of action of diazinon and chlorpyrifos, a similar mechanism (induction of metabolic activation) can be inferred for atrazine's potentiation of the acute neurotoxicity of diazinon to midges in the same study. Because the mechanism of interaction is inferred from a similar chemical, a rating of II is chosen for mechanistic understanding.

Toxicological Significance - Atrazine potentiated the acute neurotoxicity (inability of midge larvae to perform normal swimming motions) of diazinon in 96-hour static toxicity tests (Belden and Lydy 2000). Organophosphorus insecticides act as neurotoxins by inhibiting acetylcholinesterase activities in insects as well as in humans. Metabolic activation of these chemicals is similar in insects and in humans. Therefore, the result has relevance to humans. Atrazine also potentiated diazinon's inhibition of acetylcholinesterase and diazinon's acute lethality to amphipods, but did not affect diazinon's acute lethality to houseflies (Anderson and Lydy 2002). Because the mechanistic support is inferred from a atrazine's interaction with chlorpyrifos (an organophosphorus insecticide similar to diazinon), and because the potentiation was not seen in all species tested, confidence is medium and a rating of B is appropriate.

Table 3. Effect of **Simazine** on **Diazinon**: Neurological Toxicity**BINWOE: >IIB**

Direction of Interaction - The direction of interaction is expected to be greater-than-additive, based on the potentiation of diazinon neurotoxicity in the midge by a similar triazine herbicide, atrazine (Belden and Lydy 2000); potentiation of diazinon lethality and acetylcholinesterase inhibition in amphipods by atrazine (Anderson and Lydy 2002); induction of metabolic activation of a similar organophosphorus insecticide, chlorpyrifos, in midges by atrazine (Belden and Lydy 2000); and on a similar mechanism of neurotoxicity in invertebrates and humans.

Mechanistic Understanding - Diazinon is a phosphorothioate organophosphorus insecticide that is metabolically activated through oxidative desulfuration to diazoxon by cytochrome P450. Diazoxon binds to acetylcholinesterase, inhibiting its ability to hydrolyze the neurotransmitter acetylcholine. The resulting accumulation of acetylcholine at the nerve endings causes continual neurological stimulation. This mechanism of action applies to both invertebrates and mammals. Simazine and atrazine are structurally and toxicologically similar triazine herbicides. Atrazine induced the metabolic activation of a phosphorothioate organophosphorus insecticide, chlorpyrifos, and potentiated its acute neurotoxicity to midges (Belden and Lydy 2000). Based on the similarity in structure and mechanism of action of diazinon and chlorpyrifos, a similar mechanism (induction of metabolic activation) can be inferred for atrazine's potentiation of the acute neurotoxicity of diazinon to midges in the same study, and by analogy, for simazine. Because of uncertainties inherent in extrapolating from insects to humans, and because the mechanism is inferred from similar chemicals, a rating of II is chosen for mechanistic understanding.

Toxicological Significance - Atrazine, a triazine herbicide very similar to atrazine, potentiated the acute neurotoxicity (inability of midge larvae to perform normal swimming motions) of diazinon in 96-hour static toxicity tests (Belden and Lydy 2000). Organophosphorus insecticides act as neurotoxins by inhibiting acetylcholinesterase activities in insects as well as in humans. Metabolic activation of these chemicals is similar in insects and in humans. Therefore, the result has relevance to humans. Atrazine also potentiated diazinon's inhibition of acetylcholinesterase and diazinon's acute lethality, but did not affect diazinon's acute lethality to house flies (Anderson and Lydy 2002). Because the toxicological interaction is inferred from the interaction of a related chemical (atrazine) with diazinon, the mechanistic basis is inferred from related chemicals (atrazine and chlorpyrifos), and the potentiation was not seen in all species tested, confidence is medium and a rating of B is appropriate.

Table 4. Effect of **Atrazine** on **Nitrate**: Carcinogenicity
Effect of **Nitrate** on **Atrazine**: Carcinogenicity

BINWOE: >IIC

Direction of Interaction - The direction of interaction is expected to be greater-than-additive, based on the chemical interaction of atrazine and nitrite (the metabolite of nitrate) to form N-nitrosoatrazine, a more genotoxic compound. In addition, although atrazine and nitrate/nitrite are not considered carcinogenic, N-nitrosoatrazine may possibly be carcinogenic, based on carcinogenicity data for most other N-nitrosamines (including some with similar structures) and their precursors.

Mechanistic Understanding - Atrazine and nitrite (the metabolite of nitrate) react at acidic pH to form N-nitrosoatrazine (Eisenbrand et al. 1975b; Krull et al. 1980; Mirvish et al. 1991; Wolfe et al. 1976). The formation of N-nitrosoatrazine from atrazine and nitrite has been demonstrated in soil (Kearney et al. 1977), in human gastric juice (Cova et al. 1996) and in mice (Krull et al. 1980). N-nitrosoatrazine has been tentatively identified in Mississippi River water and New Orleans drinking water (Fine et al. 1976). Thus, the evidence of N-nitrosoatrazine formation is clear. The mechanistic implications for direction of interaction are not as clear because little is known regarding the toxicity of N-nitrosoatrazine relative to its precursors, other than that it is more clastogenic and mitogenic (see *Toxicological Significance*). Nevertheless, toxicological information from most other N-nitrosamines, and from *in vivo* administration of other amine compounds with nitrite indicates that the interaction of atrazine and nitrate/nitrite to form N-nitrosoatrazine is likely to be greater-than-additive with regard to carcinogenicity, and warrants a rating of II for mechanistic understanding.

Toxicological Significance - Adequate studies of the joint toxic action of atrazine and nitrate or nitrite, or of the toxicity or carcinogenicity of N-nitrosoatrazine, were not located. Studies comparing the genotoxicity of N-nitrosoatrazine with that of atrazine or nitrate provide some relevant information. Neither atrazine nor N-nitrosoatrazine caused mutations in *S. typhimurium* with or without rat liver S9 (Ishidate 1983; Ishidate et al. 1981), but rat liver S9 is generally less effective at activating N-nitrosamines than is hamster liver S9 (Lijinsky 2001) and possibly less effective than human liver S9 (Hakura et al. 2003). The clastogenicity of N-nitrosoatrazine in cultured human lymphocytes was much greater than that of atrazine or nitrate, and N-nitrosoatrazine was mitogenic but atrazine was not (Meisner et al. 1993). N-nitrosoatrazine caused chromosomal aberrations in a Chinese hamster fibroblast-derived cell line at concentrations much lower than concentrations of atrazine that gave negative results in the same study (Ishidate 1983; Ishidate et al. 1981). Thus, N-nitrosoatrazine is more clastogenic than atrazine or nitrate, and stimulates cell division whereas atrazine does not. These results indicate that the chemical interaction of atrazine with nitrite to form N-nitrosoatrazine may be a greater-than-additive interaction for genotoxicity. Although correlations between genotoxicity in mammalian cells and carcinogenicity have not been established for the N-nitrosamines, most of the 232 N-nitrosamines that have been tested for carcinogenicity are carcinogenic, including some with structures similar to N-nitrosoatrazine (Lijinsky 2001; Preussmann and Stewart 1984). Oral administration of a variety of amine compounds together with nitrite to rats and mice has resulted in the induction of tumors of the same site and type as induced by the corresponding N-nitrosamine, whereas the parent amine compounds and nitrite were not carcinogenic (Preussmann and Stewart 1984). Structure-activity considerations raise a concern for potential carcinogenicity of this nitrosamine. However, the issue of atrazine/nitrate combination and potential cancer risk in humans is still unresolved and further studies are needed.

Table 5. Effect of **Simazine** on **Nitrate**: Carcinogenicity
Effect of **Nitrate** on **Simazine**: Carcinogenicity

BINWOE: >IIC

Direction of Interaction - The direction of interaction is expected to be greater-than-additive, based on the chemical interaction of simazine and nitrite (the metabolite of nitrate) to form N-nitrososimazine, a more genotoxic compound. In addition, although simazine and nitrate/nitrite are not considered carcinogenic, N-nitrososimazine may possibly be carcinogenic, based on carcinogenicity data for most other N-nitrosamines (including some with similar structures) and their precursors.

Mechanistic Understanding - Simazine and nitrite (the metabolite of nitrate) were shown to react at acidic pH to form N-nitrososimazine (Eisenbrand et al. 1975b). The formation of N-nitrososimazine from simazine and nitrite also has been detected in rats following oral administration (Dmitrenko et al. 1996). Thus, the evidence of N-nitrososimazine formation is clear. The mechanistic implications for direction of interaction are not as clear because little is known regarding the toxicity of N-nitrososimazine relative to its precursors, other than that it is more clastogenic (see *Toxicological Significance*). Nevertheless, toxicological information from most other N-nitrosamines, and from *in vivo* administration of other amine compounds with nitrite indicates that the interaction of simazine and nitrate/nitrite to form N-nitrososimazine is likely to be greater-than-additive with regard to carcinogenicity, and warrants a rating of II for mechanistic understanding.

Toxicological Significance - Adequate studies of the joint toxic action of simazine and nitrate or nitrite, or of the toxicity or carcinogenicity of N-nitrososimazine, were not located. Studies comparing the genotoxicity of N-nitrososimazine with that of simazine provide some relevant information. Neither simazine nor N-nitrososimazine caused mutations in *S. typhimurium* with or without rat liver S9 (Ishidate 1983), but rat liver S9 is generally less effective at activating nitrosamines than is hamster liver S9 (Lijinsky 2001), and possibly less effective than human liver S9 (Hakura et al. 2003). N-nitrososimazine was clastogenic at a concentration 3-fold lower than a non-clastogenic concentration of simazine (Ishidate 1983), raising the concern that a chemical interaction between simazine and nitrite may result in a new chemical that is more clastogenic than simazine. This reasoning is supported by analogy with studies of N-nitrosoatrazine, which is more clastogenic than atrazine or nitrate, and which stimulates cell division whereas atrazine does not (Ishidate 1983; Ishidate et al. 1981; Meisner et al. 1993). These results indicate that the chemical interaction of simazine with nitrite to form N-nitrososimazine may be a greater-than-additive interaction for genotoxicity. Although correlations between genotoxicity in mammalian cells and carcinogenicity have not been established for the N-nitrosamines, most of the 232 N-nitrosamines that have been tested for carcinogenicity are carcinogenic, including some with structures similar to N-nitrososimazine (Lijinsky 2001; Preussmann and Stewart 1984). Oral administration of a variety of amine compounds together with nitrite to rats and mice has resulted in the induction of tumors of the same site and type as induced by the corresponding N-nitrosamine, whereas the parent amine compounds and nitrite were not carcinogenic (Preussmann and Stewart 1984). Structure-activity considerations raise a concern for potential carcinogenicity of this nitrosamine. However, the issue of simazine/nitrate combination and potential cancer risk in humans is still unresolved and further research is needed.

2.4 Recommendations for Data Needs

Neither *in vivo* data from human or animal studies nor *in vitro* data examining the toxicity of the four-component mixture or three-component submixtures, are available. Similarly, PBPK models describing the behavior of the four-component mixture or the three- or two-component submixtures are not available. In the absence of data for the complete mixture, a component-based approach was utilized. However, mechanistic or toxicological data pertinent to the joint toxic action of diazinon and nitrate are lacking, and data for several of the pairs are not adequate to predict the direction of interaction for some toxicities, as can be readily seen from the BINWOE matrix in Chapter 3.

For the individual components, an intermediate or chronic oral MRL is available only for diazinon, but reasonably suitable health guidance values were available for the other components. A notable data gap is the lack of adequate studies of the potential carcinogenicity of N-nitrosoatrazine and N-nitroso-simazine, chemical interaction products of atrazine and simazine with nitrate.