SUMMARY REPORT

OF REVIEWER COMMENTS

ON THE

INTERACTION PROFILE FOR

ATRAZINE, DEETHYLATRAZINE, DIAZINON, NITRATE, AND SIMAZINE

Submitted to:

The Agency for Toxic Substances and Disease Registry
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Submitted by:

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May 14, 2003

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SECTION 1

PEER REVIEWERS' SUMMARY REPORTS ON PROFILE

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Dear Mr. Lord:

As you requested, I have reviewed the Draft Interaction Profile for: Atrazine, Diazinon, Nitrate, and Simazine prepared by Syracuse Research Coordination and dated April 2003. I will first address the major issues outlined in the "Guidelines for Peeer Review of ATSDR's Interaction Profiles" that you sent to me. Then I will describe detailed comments directed to particular passages in the Draft Interaction Profile. These comments will help you discern what I was thinking when I made various marks in my often illegible handwriting on the text.

Chapter 1. Are the purposes and rationale clearly stated?

Yes. The relatively frequent presence of all these materials in well water from rural areas, the finding that the triazines should be considered a common mechanism group for possible reproductive effects, and the known reaction of nitrite (a metabolite of nitrate) with secondary amines like the traizine herbicides to form carcinogenic nitrosamines provide ample grounds for a formal evaluation of the risks posed by these mixtures. The rationale is weakest for inclusion of diazinon. Although diazinon is present with the other chemicals, and there is evidently some evidence for interaction a mechanism or a rationale for suspecting an interaction with the other components of the mixture is not clearly articulated (nor does the subsequent analysis reveal appreciable evidence for an interaction).

Chapter 2. Interactions Data for Mixtures of Concern and Component Mixtures

> **Are there any additional studies that should be considered or included in the Interactions Profile? Specifically, are there any general issues or data relevant to child health and developmental effects that have not been discussed in the profile and should be?**

One issue that I think would have been better to analyze in some detail is the likely carcinogenic activity and potency of the general body of carcinogenic nitrosamines. This is because the relatively low rating of interaction potential for nitrate/nitrite with the triazine herbicides stems in part from the lack of full blown 2 year chronic animal bioassay studies of these chemicals. Based on the history of nitrosamine carcinogenesis observations, I think that there is a very small chance that, if properly tested, nitrosoatrazine and nitrososimazine would not be found to have carcinogenic activity. Accordingly, I think that the ATSDR would be able to revise its apparent judgement of the likelihood of this interaction if it were to systematically review the substantial body of evidence from the carcinogenic testing of other nitrosamines. My expectation is that such a review would reveal that more than 95% of properly tested nitrosamines show significant carcinogenic activity, and that where mechanistic information is available, this will point strongly to an appreciable genotoxic contribution to the carcinogenesis.

Moreover, based on the apparently high level of potency in an *in vitro* clastogenesis assay reported by Meisner et al. (1993) for nitrosoatrazine, I think there is a reasonably good chance that these compounds will turn out to be relatively potent nitrosamines. The abstract of this paper reads in part:

> "Exposing human lymphocyte cultures to concentrations of N-nitrosoatrazine (NNAT) as low as 0.0001 microgram/ml results in significant elevations in chromosome breakage as well as an increased mitotic index. In contrast, 1,000-10,000-fold greater concentrations of nitrates, nitrites, and/or atrazine was required to produce comparable chromosome damage and, in those cases where the mitotic index was affected, it was decreased."

This material is referred to on pp. 10-11 of the current document. Therefore I think it would also contribute to the analysis to document the potency of a substantial number of nitrosamines, both in chronic 2-year carcinogenesis bioassays and in this type of in vitro human cell clastogenesis assay. This would allow ATSDR to construct an a priori distributional analysis for the apparent potency of this nitrosamine relative to other nitrosamines.. (By potency, I mean they would cause a given incidence of cancer or chromosome breakage at a relatively low exposure level.)

A similar kind of review of the relative potency of other nitrosamines in fetal and early postnatal periods relative to adults would shed light on potential fetal and child sensitivities for nitrosoatrazine and nitrososimazine.

Reference: Meisner, L. F., Roloff, B.D., and Belluck, D. A. "In vitro effects of N-nitrosoatrazine on chromosome breakage." *Archives of Environmental Contamination and Toxicology* 24(1): 108-112 (1993).

Are there epidemiology studies that support the interaction data and assist in the evaluation of interactions and joint toxicity?

Not that I am aware of. The document seems to do a relatively good job of skeptically reviewing the sparse available epidemiological information on these issues.

Are the studies described in enough detail to support ATSDR's interpretations

I think so, with the exception of the expanded analyses I suggested above for the nitrate/nitrite with triazine herbicide interactions.

Are the tables easy to understand and consistent with the text?

Yes, to the extent that it was easy to understand what was done and how the numerical conclusions were arrived at. The numerical system for rating evidence for interaction potential on the basis of the kinds of studies that are directly available for mixture components, however, I think has some fundamental flaws that are revealed by the nitrate/nitrate triazine herbicide example. The gross kind of experimental evidence (in vitro, animal in vivo, human epidemiology) is not, as implied by the system in its present form, an infallible guide to how strong the evidence is for a particular kind of mechanistic interaction. In general I think that the reviewers need to consider a wider range of evidence bearing on the plausibility of the interactions that are being evaluated. In this case ATSDR should review and give much more weight to the implications of the overwhelming volume of data on nitrosamine carcinogenesis and the production of nitrosamines in vivo, in vitro, and in the environment by the reaction of secondary amines and nitrite.

Was the most appropriate evaluation method selected?

No—see previous comment.

If employed in the Interactions Profile, please evaluate WOEs independently to determine if conclusions match those of the authors.

I have no quarrel with the authors' conclusions in the case of the likely additive interactions for the triazines with respect to developmental effects; or for the absence of likely interactions for the cholinesterase-inhibition effects. I think the authors have arrived at a serious underestimation of the much greater than additive interaction between nitrate/nitrite and the triazine herbicides, which I would consider very highly likely (say 0.49-0.79, rather than 0.08 on the overall numerical scale used in the document—see calculations and futher discussion on p. 6 below).

Do the conclusions drawn have a direct impact on health assessments of populations exposed to chemicals discussed in the document?

Yes. Clearly additively-combined weighted potency analysis is indicated for the reproductive effects. But I would suggest much more extensive analysis of the degree to which nitrate/nitrite in waster will give rise to increased exposure to nitrosoatrazine and nitrososimazine, and quantitative distributional evaluation of the potential carcinogenic risks arising from exposure to those compounds. (By a distributional evaluation I mean both a quantitative uncertainty analysis based in part on the uncertainties in exposure and carcinogenic potency, and a variability analysis including age-related differences in both exposure and likely potency based on experience with other genetically-acting carcinogens in animals and humans). This will require substantial additional analysis, and it would also, of course, be desirable to collect better data both on the exposure and the cancer potency side.

Chapter 3. Recommendations for Exposure-based Assessment of Joint Toxic Action of the Mixture

Are recommendations consistent with information provided in the appendices and Chapter 2?

Yes, as far as I can tell. Of course, as outlined earlier, I disagree with some of the evaluations in Chapter 2

Are the recommendations useful to public health officials?

I think the recommendations for the reproductive effects are useful to the extent they recommend additivity. I am not entirely convinced that public health officials will find the numerical BINWOE information fully comprehensible or useful. And I think public officials are likely to be misled, if anything, by the low numerical ranking of the likelihood of greater than additive interaction between nitrate/nitrite and the triazine herbicides.

Appendices

Does the information provided in each appendix support the data provided in Chapter 2?

Yes, as far as I can tell.

Are there additional data supporting the Interactions Profile that should be included? If so, please provide references.

As I indicated earlier, I think ATSDR should compile the available results of 2-year cancer bioassays for nitrosamines to document (1) the frequency with which adequately tested agents in this chemical group are found to have carcinogenic activity, and (2) the distribution of cancer potencies in this group of chemicals. A similar compilation of potencies should be done for the human lymphocyte clastogenicity assays for comparison with the Meisner et al. (1993) observations cited earlier. I don't know of previous analyses that do these jobs, nor can I compile the extensive literature in these fields within the limited scope of this review.

Detailed Comments on Specific Portions of the Text

Summary, page v, 3rd par. I don't think that the guidance that two or more components of a mixture should exceed a hazard index of 0.1 before a significant interaction is suspected should be applied to this case. The interaction between nitrate/nitrite and the triazine herbicides is expected to generate novel chemicals (nitrosamines) whose mode of toxic action is completely different from the interacting chemicals. Therefore the MRL's or RfD's for the triazine and nitrate/nitrite components are completely irrelevant and potentially misleading as a basis for judging when an interaction has the potential to pose a significant public health risk.

p. 2, 2nd to last sentence of the second new paragraph. I don't understand the comment that "the lowest-observed-adverse –effect level (LOAEL) for both durations was lower than applicable no-observed-adverse-effect levels (NOAELs), and was considered a serious LOAEL for reproductive effects (including anestrus)." If observed LOAELs for some experiments are lower than the corresponding NOAELs then the LOAELs should become the basis for RfDs and MRLs, and the NOAELs from the less sensitive experiments become irrelevant. Some revision or clarification seems in order.

p. 3, end of par continued from previous page—Some quantitative SAR analysis results would be of interest to cite if they are available. Otherwise the document should clarify that the "SAR" in this case is just the general observation that most nitrosamines show carcinogenic activity when adequately tested. Elsewhere I have recommended a quantitative analysis of this at two levels (1) incidence of positive findings among adequately tested nitrosamines, and (2) a distribution of observed cancer potencies.

p. 5, 1st par under section 2.2.1—I don't know what "salmon parr" are. A brief parenthetical definition would help.

p. 6, 2nd par—the quantitative relationship of chromosome breakage to the coefficient of variation of the G1 peaks is quite unclear. Perhaps some supplementary background data or illustration would be helpful here.

p. 7, first paragraph. The authors explain well here and later that the mechanism of reproductive senescence is different between rats and people. However they do not provide the evidence that rat reproductive senescence is causally related to the observed increase in carcinogenesis associated with the triazine herbicides. This is plausible because of the well known carcinogenic effects of increased estrogen levels (in people as well as rats). However it seems to me that the case for concluding the mechanism is via the acceleration of rat reproductive senescence and that this is totally unrelated to possible breast cancer enhancement in humans (say, prior to human menopause) nis not fully made here.

p. 8, 1st 2 paragraphs under section 2.2.4. The authors should attempt to integrate the prior in vitro and soil observations to model the processes of formation and destruction of nitrosoatrazine in different media in water, soil, and gastric contents for human babies and adults. In this way, readers might get something more than a qualitative indication of possible exposure; it would be highly desirable to have some basis for developing quantitative expectations, given the concentrations of reactants, time, temperature, and pH. This is not a new problem. I suspect that fruitful analogies can be made using observations of the kinetics of formation (and possibly destruction) of other nitrosamines.

p. 11, middle of 1st new paragraph—The report of the percentage of atrazine and nitroatrazine metabolized in vitro is not of tremendous significance, but for completeness the authors should say what time span was involved in the observations—a % metabolized in vitro without saying over what period is essentially meaningless.

p. 18 and p. 19, based on analogies with the body of other nitrosamines I would change the ratings for mechanistic understanding and toxicological significance to I or at least II. Therefore the numerical calculation should be at least (1 X 0.79 X 0.79 X 0..79) = 0.49 and possibly as high as (1 X 1 X 1 X 0.79) = 0.79. I also think it is baloney to say that the "mechanism of action of this compound is unknown." While the information may well be sparse for these particular compounds, I think the inference is very much in order that primary genotoxic action is highly likely based on the extensive information we have on the metabolism of other nitrosamines to genetically activated reactive intermediates and widespread observations of mutagenic and clastogenic activity in a variety of test systems.

p. 20 Similar comments are in order on the necessity to use information from other nitrosamines are in order for comments made in the "recommendations of data needs" section. Tests should surely be done on nitrosoatrazine and nitrososimazine themselves, but the likely results of these tests can hardly be in much doubt.

p. 21, 2nd par. This is where I noted the opportunity to do some comparative distributional analyeses of carcinogenic and clastogenic potency. Together with chemistry modeling results to

predict the amounts of the nitrosamines formed in drinking water and in gastric contents, the results would guide managers and analysts on how seriously to take mixtures of various concentrations of nitrate/nitrite and the triazine herbicides.

pp. 22-23. I disagree that the confidence should be considered low in the greater-than-additive predictions for carcinogenicity between nitrate/nitrite and the triazine herbicides.

p. 24. As indicated earlier, I would raise the nitrate/triazine herbicide interaction ratings to between +0.49 and +0.79.

p. 25—Similarly I would revise the description of confidence in the conclusions. Data on nitrosamines in general are, I think, more than adequate to draw inferences about the likely carcinogenic risks of nitrosoatrazine and nitrososimazine, even though the evidence from direct testing of these compounds themselves is quite limited.

p. 52, par 4 under "Health Guidelines". It is not at all clear why the authors (and, reportedly EPA) believe that "No additional FQPA safety factor was needed" for cholinesterase inhibition. As a member of the FQPA science advisory board, I can say that when we reviewed EPA's proposed risk assessment of cholinesterase-inhibiting pesticides we concluded that the FQPA mandate for a 10-fold safety factor unless adequate evidence was available to show safety for the developing nervous system was not satisfactorily overcome by EPA's 2002 analysis of developmental effects for the organophosphate common-mechanism group. My own reason for supporting this conclusion that the FQPA factor must be retained is that even if cholinesterase inhibition is below detectable levels in the mother of a developing fetus or an early neonate, it is still quite possible that a modest inhibition of acetylcholinesterase activity could reinforce some neural pathways relative to others and lead to long-lasting changes that are specific to the times when particular neural connections are competing with one another for survival.

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Dr. Kannan Krishnan's Review of the Interaction Profile for Atrazine, Deethlyatrazine, Diazinon, Nitrate and Simazine

GENERAL COMMENTS

This document presents a systematic evaluation of available data on the joint action of the above chemicals in view of recommending approaches for exposure-based assessment of potential hazard to public health. The authors conclude, based on available evidence, that the assessment for this mixture should consider the (i) additive joint reprotoxicity of atrazine and deethylatrazine (high confidence), (ii) the neurotoxicity of diazinon (medium-low confidence, due to the possible potentiating effect of triazines), (iii) hematotoxicity of nitrate (high uncertainty due to lack of knowledge regarding the effect of diazinon and triazines), and (iv) possible carcinogenic effect of the N-nitroso compounds formed from triazines and nitrate (low confidence due to lack of bioassay data). The preceding conclusions are consistent with the current knowledge base regarding the above chemical mixture.

The main text of the document presents an appropriate review of the available studies (or identifies the lack of relevant data) with respect to the health effects of the mixture taken as a whole or as binary combinations of components. The assessment endpoints for this mixture, direction of known interactions among the components and the relevance of the joint action data for exposure-based assessments, suggested in the document are scientifically-sound. The presentation of available data throughout this document is concise and clear. The interpretations and recommendations are consistent with the studies evaluated. The appendices provide appropriate but very brief background information on the toxicokinetics, health effects, mechanisms of action, health guidelines and target organ toxicity dose (where available) for each mixture component. However, the reviewer is concerned about the TTD values reported for deethylatrazine and simazine. Some missing references included with this report may be used to strengthen the document.

Overall, this document is a very good example of how interaction profiles should be developed for mixtures of concern - on the basis of critical effects of components and relevant joint toxicity information. This document should be useful to public health officials and general public concerned about the health risks of combined exposure to atrazine, deethylatrazine, diazinon, nitrate and simazine.

REVIEWER RESPONSE TO PROFILE-SPECIFIC QUESTIONS

Chapter 1. Introduction

Are the purpose and rationale clearly started ?

Yes. However, on page 3, some of the conclusions of mixture studies are presented (even before being described in detail), thus confusing the reader at this stage.

Chapter 2. Interactions data for mixture of concern and component mixtures

Are there additional studies that should be considered in the Profile ?

Regarding the whole mixture, an NTP study conducted using a mixture of atrazine, nitrate, simazine and certain other contaminants (representative of groundwater contaminantion in Iowa and California) would be relevant. I am enclosing a summary of findings reported by RSH Yang that is available in the internet. In this study, sister chromatid exchanges would appear to have been marginally increased in rats and mice receiving the California mixture (i.e., atrazine, nitrate, simazine and certain other contaminants). No other significant changes were observed in this 26-week study (See **ATTACHMENT 1**).

An in vivo study on simazine nitrosation exists in the literature and can be cited (**ATTACHMENT 2**).

There is an abstract that supports the authors' statement on the low confidence regarding the carcinogenicity of N-nitrosoatrazine (Weisburger et al. 1990). Even though I could not obtain a copy of this abstract, I am including a statement of the observations reported in that abstract (**ATTACHMENT 3**).

Belden and Lydy's observations are further corroborated by another report of more-than-additive toxicity of diazinon and atrazine (Anderson and Lydy 2002, **ATTACHMENT 4**).

Are there epidemiology studies that support the interaction data and assist in the evaluation of interactions and joint toxicity ?

Not that I know of. But there are quite a lot of epidomiological studies investigating the relationship between health effects and exposure to atrazine and other agricultural contaminants. I don't think a comprehensive review of

such studies is needed or would help in evaluating the extent of interactions and joint toxicity of specific chemicals.

Are the studies described in enough detail to support ATSDR's interpretations ?

Yes, except for that of Belden and Lydy (2000) on page 7. The exposure concentrations should be specified here.

Are the tables easy to understand and consistent with the text ?

Yes. However, Table 6 should indicate that the assessments should take into account the N-nitroso compounds resulting from chemical-chemical interactions.

In Table 7, the numerical value for atrazine on diazinon should be 0.23 (to be consistent with the text on page 16).

Was the most appropriate evaluation method selected ?

Yes

If employed in the interactions profile, please evaluate WOEs independently to determine if conclusions match those of the authors.

1. Simazine on atrazine/deetylatrazine (page 15)

My evaluation is identical to that of the authors

2. Atrazine/dethylatrazine on diazinon

I would give 0.71 for toxicological significance, since it can be inferred or has been demonstrated for related chemicals. The arguments "one relevant study" and "insect-human differences" are irrelevant here. When the dose levels are appropriate, it is clear that such a potentiation will occur – that is the inference from the study with related chemicals. So the score for toxicological significance should be 0.71 (i.e., B).

On page 16, para 1, ...induction by diazinon....should read .....induction by atrazine.

3. Simazine on diazinon

Same as above (0.71), because, in this case, the toxicological consequence can be inferred from atrazine-chlorpyriphos combination. The arguments of one relevant study and insect-human differences are not relevant to the choice of the score per information presented on page 14.

On page 17, para 1, ...induction by diazinon....should read .....induction by atrazine.

4. Atrazine and nitrate

I tend to think that, for mechanistic understanding, the score should be 1 because the mechanism by which the interaction occurs is well characterized. There is no doubt here. It is unclear as to why one needs to have a mechanistic understanding of the action of the chemical to have a greater rating here. Is n't it the mechanistic understanding of the interaction and not the mechanistic understanding of the action of the chemical itself, that is important here. The confidence, of course, is low concerning the activity of N-nitrosoatrazine/simazine compounds. Therefore, the choice of C for toxicological significance in this case is excellent. But again, the modifying factor should be 1 since the formation seems to have been demonstrated in vivo (e.g., page 9).

5. Simazine and nitrate

See comments above.

Do the conclusions drawn have a direct impact on health assessments of populations exposed to chemicals discussed in the document ?

The final sentence on page vi summarizes this aspect appropriately.

Chapter 3. Recommendations for exposure-based assessment of joint toxic action of the mixture

Are recommendations consistent with information in the appendices and chapter 2 ?

Yes

Are recommendations useful to public health officials ?

Yes

Appendices

Does the information provided in each appendix support the data provided in Chapter 2?

Yes

Page 42, line 1

..the chronic Deethylatrazine..... should read....the chronic PAD...

TTD values for deethylatrazine

Why is deethylatrazine and atrazine assumed to have equivalent toxicity – on the basis of mg/kg/d. This assumption is valid only if 100% of atrazine is converted to deethylatrazine. Otherwise, the percent difference should be reflected in the TTD.

Further, 0.0018 mg of atrazine is not equal to 0.0018 mg deethylatrazine, even if we assume absolute toxic equivalency. The equivalency conversions are best done on the basis of moles, and then they can be converted and expressed in mg.

TTD values for simazine

0.0018 mg atrazine and 0.0018 mg simazine will not be equivalent since molecular weights are different (215.69 *vs* 201.69). This should be taken into account in establishing the TTD for simazine from that of atrazine.

TTD for diazinon

Why is the intermediate oral MRL value not specified as the TTD value ? That value should be cited as the summary TTD value, as done for other chemicals in this document.

Are there additional data supporting the interactions profile that should be included ?

No.

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Dear Mr. Lord:

As requested, enclosed is my peer review of the "Interaction Profile for Atrazine, Deethylatrazine, Diazinon, Nitrate, and Simazine." Only those pages with corrections are included herewith, and following those pages I have my written comments as a separate section. In summary, the document satisfies the needs for health assessors to reach regulatory decisions.

Per your request, I have also enclosed the invoice for your records.

I hope this review is satisfactory. If you have any questions or comments, please feel free to call me at my office (541) 737-9036, or my home (541) 754-1028.

Yours sincerely,

Sheldon L. Wagner M.D

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Enclosure

REVIEW OF:

INTERACTION PROFILE FOR: ATRAZINE, DEETHYLATRAZINE, DIAZINON, NITRATE, AND SIMAZINE

by

Sheldon L. Wagner, MD

Oregon State University

Page V:

The notations here were made but the second notation effectively answers the first question, in which I noted that the reasoning didn't flow together. Nevertheless, I do not recommend a change on this page.

Page 1:

I believe the standard at this time was 40 ppm, and, if so, this should be stated in order to clarify this section.

Page 2:

This was an excellent review for interested readers who wish to know more detail.

Page 3:

I think the word "suspicion" would be better if it was replaced by a phrase such as "considerations raise the scientific issue that further studies need to be performed to determine the carcinogenic potential."

Page 5:

Would you care to also add the statement that similar effects have been found with other chemicals, such as chlorpyrifos. This is just a matter of interest, and perhaps does not need to be added in this section.

Page 6:

This is a good review, again, which supports your conclusions.

Page 7:

Upper statement. Pointing out that is an excellent review.

Lower statement leads me to question whether you wish to add the fact that the olfactory effect, which is seen in atrazine and simazine, may not be seen with diazinon because it hasn't been studied; however, this effect has been seen with other organophosphates such as chlorpyrifos.

Page 9:

Regarding the study about cancer and drinking water contamination with atrazine, it was my professional opinion that this was considered an important study, regardless of the shortcomings. Perhaps a statement to that effect may be valuable since otherwise the paragraph stands alone without, and I would prefer some emphasis.

Page 11:

I believe the sentence could be changed to: "This raises a concern about unresolved scientific issues about the formation of N-nitrosoatrazine....."

Page 15:

It is my feeling that the species difference therefore decreases the potential for the toxicological significance of this effect and should be stated.

On the last line, I question whether or not the rating should be B rather than A.

Page 16:

I am simply questioning whether a rating of B would be more appropriate than a rating of C?

Page 21:

The sentence outlined appears incorrect. It seems to me that if a health hazard is exceeded by one component, no further assessment is needed because a health hazard is already established. Therefore, the term "unlikely" seems inappropriate in the sentence which I have highlighted.

Page 22:

The first comment is correct, although I would add that they are unique to diazinon, as well as other organophosphates.

The second comment is one in which I question whether such data exist. If it does not, and I do not believe it does, this fact should be stated.

Page 38:

In the upper paragraph, would you care to make a statement that therefore, one can conclude that the studies are weak?

In the next paragraph, I question whether you wish to correlate levels of atrazine found in the environment versus those found in experimental animal studies, such as described. At the least, the levels in such animal studies should be stated.

At the bottom of this page, as above, you'll note that I'm asking you for a relative dose of exposure so that some consideration by the interested reader could be made, rather than having to search through the paper as it stands to find the necessary data.

Page 39:

In the top paragraph, you make a statement of strength of the studies which is a good conclusion.

In the second comment, I am looking for relative dose in order to assist the reader at this point, rather than having to go through the Table of Contents and search for the necessary route numbers and dose.

Page 40:

Again, this is a question of relative dose. At the very least, animal studies should give the dose so that one can compare against the environment at some point.

Page 41:

My comments here refer to the importance of the FQPA and its additional safety factor of 10 for infants and children. I would at least cite where the FQPA law can be found, either on the Internet or elsewhere.

Page 46:

I am again questioning whether the exposure/dose should be given here so that one does not have to search through the paper for the specific data.

Page 49:

I believe the second bullet would be better if it were changed to "cleavage of the ester bonds to alkylphosphates of diazinon such as...."

On the second comment, I would change the wording "to diazoxon" at the point noted.

Page 50:

At the end of the top paragraph, I would add the sentence: "Continuous stimulation of the nervous system at the synapse occurs."

In the next paragraph, I have highlighted "vasodilation and hypotension." If you have good documentation of this, I would find it acceptable; however, a peculiarity of organophosphate intoxication in general is that occasionally it produces a significant hypertension, and therefore I would not include the words about vasodilation and hypertension without this qualification.

Further down, after the words "depending on the extent," I would add "and route," since the route of exposure is quite important with organophosphate intoxication.

The last comment is that I would add the words "the syndrome principally affects the upper extremities and cranial nerves." This is an important point because the typical organophosphate intoxication usually begins in the lower extremities.

Page 52:

I would replace the word "needed" with the word "used."

Page 58:

In expressing the TTD, I believe it would be helpful to the reader, in addition to the dose of 1.6 mg/kg/day, if you would consider adding the levels in parts per million (ppm). The common interchanging of these terms in toxicology is confusing. In the case of nitrates, regulations are based not as much upon TTD as they are about ppm in the water or foods, etc.

SECTION 2

ADDITIONAL REFERENCES AND DATA SUBMITTED BY THE PEER REVIEWERS

ADDITIONAL REFERENCES AND DATA SUBMITTED BY

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☐ 1: Toxic Rep Ser 1993 Aug;36:1-G3 Related Articles, Link

NTP technical report on the toxicity studies of Pesticide/Fertilizer Mixtures Administered in Drinking Water to F344/N Rats and B6C3F1 Mice.

Yang R.

Toxicity studies were performed with pesticide and fertilizer mixtures representative of groundwater contamination found in California and Iowa. The California mixture was composed of aldicarb, atrazine, 1,2-dibromo-3-chloropropane, 1,2- dichloropropane, ethylene dibromide, simazine, and ammonium nitrate. The Iowa mixture contained alachlor, atrazine, cyanazine metolachlor, metribuzin, and ammonium nitrate. The mixtures were administered in drinking water (with 512 ppm propylene glycol) to F344/N rats and B6C3F1 mice of each sex at concentrations ranging from 0.1x to 100x, where 1x represented the median concentrations of the individual chemicals found in studies of groundwater contamination from normal agricultural activities. This report focuses primarily on 26-week toxicity studies describing histopathology, clinical pathology, neurobehavior/neuropathology, and reproductive system effects. The genetic toxicity of the mixtures was assessed by determining the frequency of micronuclei in peripheral blood of mice and evaluating micronuclei and sister chromatid exchanges in splenocytes from female mice and male rats. Additional studies with these mixtures that are briefly reviewed in this report include teratology studies with Sprague-Dawley rats and continuous breeding studies with CD-1 Swiss mice. In 26-week drinking water studies of the California and the Iowa mixtures, all rats (10 per sex and group) survived to the end of the studies, and there were no significant effects on body weight gains. Water consumption was not affected by the pesticide/fertilizer contaminants, and there were no clinical signs of toxicity or neurobehavioral effects as measured by a functional observational battery, motor activity evaluations, thermal sensitivity evaluations, and startle response. There were no clear adverse effects noted in clinical pathology (including serum cholinesterase activity), organ weight, reproductive system, or histopathologic evaluations, although absolute and relative liver weights were marginally increased with increasing exposure concentration in both male and female rats consuming the Iowa mixture. In 26-week drinking water studies in mice, one male receiving the California mixture at 100x died

during the study, and one control female and one female in the 100x group in the Iowa mixture study also died early. It could not be determined if the death of either of the mice in the 100x groups was related to consumption of the pesticide/fertilizer mixtures. Water consumption and body weight gains were not affected in these studies, and no signs of toxicity were noted in clinical observations or in neurobehavioral assessments. No clear adverse effects were noted in clinical pathology, reproductive system, organ weight, or histopathologic evaluations of exposed mice. The pesticide/fertilizer mixtures, when tested over a concentration range similar to that used in the 26-week studies, were found to have no effects in teratology studies or in a continuous breeding assay examining reproductive and developmental toxicity. The California and Iowa pesticide mixtures were tested for induction of micronuclei in peripheral blood erythrocytes of female mice. Results of tests with the California mixture were negative. Significant increases in micronucleated normochromatic erythrocytes were seen at the two-highest concentrations (10x and 100x) of the Iowa mixture, but the increases were within the normal range of micronuclei in historical control animals. Splenocytes of male rats and female mice exposed to these mixtures were examined for micronucleus and sister chromatid exchange frequencies. Sister chromatid exchange frequencies were marginally increased in rats and mice receiving the California mixture, but neither species exhibited increased frequencies of micronucleated splenocytes. None of these changes were considered to have biological importance. In summary, studies of potential toxicity associated with the consumption of mixtures of pesticides and a fertilizer representative of groundwater contamination in agriculturative of groundwater contamination in agricultural areas of Iowa and California failed to demonstrate any significant adverse effects in rats or mice receiving the mixtures in drinking water at concentrations as high as 100 times the median concentrations of the individual chemicals determined by groundwater surveys. NOTE: These studies were supported in part by funds from the Comprehensive Environmental Response, Compensation, and Liability Act trust fund (Superfund) by an interagency agreement with the Agency for Toxic Substances and Disease Registry, U.S. Public Health Service.

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Study of simazine nitrosation in rats.

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Source: DOPOVIDI NATSIONAL'NOYI AKADEMIYI NAUK UKRAYINY; 0 (1). 1996. 93-95.

Abstract:

BIOSIS COPYRIGHT: BIOL ABS. C14-**simazine** being introduced into **rats**, only trace amounts of labelled N-nitrososimazine were found in the liver, thymus, kidney and spleen tissues in single cases. Introduction of **simazine** together with sodium **nitrite** has lead to the considerable increase of the frequency of cases and amount of N-nitrososimazine formation in analyzed tissues. Nitrosation of C14-**simazine** in tissues, especially liver, is much more expressed as affected by injection of the BCG vaccine than under the combined introduction of this pesticide with sodium **nitrite**. The results obtained indicate the possibility of **simazine** nitrosation and accumulation of N-nitrososimazine in **rat** tissues under arrival of this pesticide into the organism together with sodium **nitrite** or after the induction of endogenic synthesis of nitric oxide (NO) induced by injection of the BCG vaccine into animals. This should be taken into account when estimating the danger of pesticides and other nitrogen-containing xenobiot

Weisenburger, D. D., Hickman, T. J., Patil, K. D., Lawson, T. A., and Mirvish, S. S. (1990). Carcinogenesis tests of atrazine and N-nitrosoatrazine-compounds of special interest to the Midwest, Proceedings of the American Association for Cancer Research, Vol. 31 [abstract 603]. Carcinogenesis, pp. 102.

ATTACHMENT #3

WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

PC Code: 080803

DATE: April 10, 2002 **DP Barcode**:

SUBJECT: EFED Review of Public Comments in Response to the EPA EFED Revised Environmental Risk Assessment for Atrazine

TO: Kimberly Lowe, Chemical Review Manager
Special Review and Reregistration Division 7508C

FROM: William Rabert, Biologist
James Lin, Environmental Engineer
Mary Frankenberry, Statistician
Douglas Urban, Senior Ecologist
Environmental Risk Branch 3
Environmental Fate and Effects Division 7507C

THRU: Kevin Costello, Acting Chief
Environmental Risk Branch 3
Environmental Fate and Effects Division 7507C

The Environmental Fate and Effects Division (EFED) has reviewed many of the public comments in response to the Notice of Availability of Environmental Fate and Effects Assessment on Atrazine to Re-registration Eligibility Decision [OPP-34237A]. Comments from nine groups were considered by SRRD to address specific or general scientific issues and were referred to EFED for response. The following contains both summary and specific comments, and EFED's responses are listed below.

American Water Works Association (AWWA) Comment: AWWA expressed concerns that atrazine would contaminate ground and surface water, especially sources of drinking water.

EFED Response: The EFED and HED science chapters address the analysis of different studies of atrazine in surface and ground water, especially in sources of drinking water. These results and areas of uncertainty are discussed extensively.

AWWA Comment: AWWA indicated that they would like to understand the Agency's rationale for coordinating the different methodologies.

EFED Response: OPP and OW managers concluded that since the two offices were working

gonadal abnormalities (personal communication, March 13, 2002). As with many field monitoring studies, when a variety of chemicals are present or have been used at a site, it is difficult to assign causation of effects to one or more of the chemicals. As indicated by the commenters, alachlor and metolachlor (both endocrine disruptors) are used in the same areas as atrazine. While there is uncertainty about which chemical or chemicals caused the effects, the field survey indicates that the gonadal effects observed in the laboratory also occur in wild populations in pesticide-treated areas. It is unclear whether the affected frog populations in these sites are capable of reproduction or if the population is totally dependent on immigration from unaffected populations. Again, it is important to demonstrate that the effects found on male gonadal development and larynges have an effect on survival and/or reproduction. Dr. Hayes indicated that the **preliminary test results** show that males with effects on gonadal development produce no sperm (personal communication, March 13, 2002). EFED has expressed interest in the ongoing research on these frogs to answer the questions on reproduction.

p. 9. **NRDC & WWF Comment**: "Second, the reproductive effects observed in frogs indicates that there may be no clear threshold for the effects of atrazine on sexual differentiation in amphibians, making exposures at current environmental levels an imminent hazard to wildlife and to endangered species."

EFED Response: The study provided with this comment reported that there was a no effect level for atrazine effects on male *Xenops* gonadal abnormalities at 0.01 μg/L. Atrazine effects on frog gonadal development have been demonstrated in the laboratory. Similar effects have been found in *Rana pipins* in areas were atrazine and other endocrine-disrupting pesticides have been used. What is not certain at this time is whether the gonadal abnormalities have an effect on reproduction. Extrapolation from atrazine effects on an amphibian to an "imminent hazard to wildlife and to endangered species" raises concerns for risks, but the warning is premature until there is credible evidence that these effects are affecting the reproduction of amphibians.

p. 11. **NRDC & WWF Comment**: "Aside from any direct carcinogenic actions of atrazine, there is evidence that the herbicide may interact with nitrate fertilizers in the environment to form a more potent carcinogen, N-nitrosoatrazine (NNAT). Weisenburger *et al.* found that NNAT is readily formed when atrazine is combined with nitrite in acid conditions in the soil or in the stomach. The authors concluded that, given the frequent coexistence of atrazine with nitrate fertilizers in agricultally contaminated water, the potential carcinogen NNAT may be a common exposure accompanying atrazine use. Therefore, NNAT formation may be an underlying mechanism in the initiation of atrazine-associated non-Hodgkin's lymphoma.

In 1993 Meisner *et al.* tested NNAT on humans to assess its genotoxicity. When human lymphocytes were exposed to very levels of NNAT (concentrations as low as 0.0001 micrograms/ml) chromosome damage was induced. The authors concluded that 'the increased incidence of stomach cancer, leukemia and lymphoma in farmers, who have the greatest exposure to both nitrates and atrazine, raises concerns about the safety of water supplies that contain both of these contaminants.'"

HED Response: HED appreciates the seriousness of this comment, and acknowledges that N-nitrosoatrazine has not been included in the risk assessment for atrazine. OPP focused the atrazine risk assessment on the significant known hazards (endocrine disruption) and exposure pathways (drinking water) associated with atrazine for which reliable exposure data were available. In particular, OPP was careful to incorporate the chlorometabolites into the assessment, and considered them to be of equivalent toxicity to the parent compound.

Since N-nitrosoatrazine can be formed *in vitro* when atrazine and nitrite are mixed at an acid pH (Wolfe, et al., 1976), and because atrazine and nitrites can occur together in drinking water, it has been hypothesized that it is possible that N-nitrosoatrazine could be formed at acid pH in the stomach. However, formation of N-nitrosoatrazine *in vivo* has not been demonstrated. N-nitrosoatrazine has been shown to be mutagenic in genotoxicity tests, but cancer bioassays in female mice and rats failed to show a carcinogenic response following N-nitrosoatrazine exposure (Weisenberger, 1990 - abstract). OPP is exploring the extent of this compound's presence in drinking water with the OW and the registrant.

EFED Response: *In vitro* effects, such as chromosome damage to human lymphocytes, are difficult to extrapolate to effects on whole organisms due to the difference in the route of exposure and the uncertainty as to the magnitude of that exposure. There is also uncertainty as to what that effect might have on survival, reproduction and/or population effects, which are EFED toxic endpoints. While N-nitrosoatrazine has been shown to be mutagenic in genotoxicity tests (i.e., human lymphocytes), cancer bioassays in female mice and rats have failed to show carcinogenic responses following treatment with N-nitrosoatrazine.

p. 11. **NRDC & WWF Comment**: "Chlorinated atrazine metabolites act as endocrine-disrupting agents in aquatic amphibians, small mammals, and humans, causing abnormal reproductive organ development and cancers of the reproductive organs. The EFED risk assessment discusses briefly the toxicity of the degradates, compared to parent atrazine (EFEC [sic], p. 41-42). The Assessment [sic] notes that toxicity data for the degradates is not available for birds, fish, aquatic invertebrates, terrestrial plants, and acute oral mammals. This is a very serious data gap, given that the degradates are long-lived, and available data indicates that they are more chronically and acutely toxic than the parent atrazine (EFEC [sic], p. 42)."

EFED Response: The commenter has not specified chlorinated atrazine metabolite(s) of concern or provided any citation showing their endocrine-disrupting effects in aquatic amphibians or small mammals. EFED has not seen any wildlife toxicity studies for the above effects due to chlorinated atrazine metabolites. Acute and chronic mammalian toxicity data were available for some atrazine degradates. The base set acute ecotox tests have been identified as data gaps for the three major atrazine degradates (i.e., hydroxyatrazine, deethylatrazine, and deisopropylatrazine). Additional acute and chronic tests are held in reserve depending on the toxicity levels found in these acute tests.

Endpoints in ecological risk assessments have been limited to mortality and reproduction effects that pose a "significant adverse effect" to local, regional and national wildlife populations. If a

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ATTACHMENT #4

INCREASED TOXICITY TO INVERTEBRATES ASSOCIATED WITH A MIXTURE OF ATRAZINE AND ORGANOPHOSPHATE INSECTICIDES

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Abstract—This study examined the joint toxicity of atrazine and three organophosphate (OP) insecticides (chlorpyrifos, methyl parathion, and diazinon) exposed to *Hyalella azteca* and *Musca domestica*. A factorial design was used to evaluate the toxicity of binary mixtures in which the lethal concentration/lethal dose (LC1/LD1, LC5/LD5, LC15/LD15, and LC50/LD50) of each OP was combined with atrazine concentrations of 0, 10, 40, 80, and 200 μg/L for *H. azteca* and 0, 200, and 2,000 ng/mg for *M. domestica*. Atrazine concentrations (≥40 μg/L) in combination with each OP caused a significant increase in toxicity to *H. azteca* compared with the OPs dosed individually. Acetylcholinesterase (AChE) activity also was examined for the individual OPs with and without atrazine treatment. Atrazine in combination with each of the OPs resulted in a significant decrease in AChE activity compared with the OPs dosed individually. In addition, *H. azteca* that were pretreated with atrazine (≥40 μg/L) were much more sensitive to the OP insecticides compared with *H. azteca* that were not pretreated with atrazine before being tested. Topical exposure to atrazine concentrations did not significantly increase OP toxicity to *M. domestica*. The results of this study indicate the potential for increased toxicity in organisms exposed to environmental mixtures.

Keywords—Atrazine Organophosphates Acetylcholinesterase *Hyalella azteca* *Musca domestica*

INTRODUCTION

The triazine-class herbicide atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine) is currently one of the most frequently used pesticides in the Midwestern agricultural regions of North America [1,2]. With over 85 million pounds applied annually, atrazine accounts for approximately 60% of the total mass of pesticides used each year [1–3]. Used to control a variety of annual broadleaf and grassy weeds, atrazine is extensively applied to corn and other silage crops as well as on a variety of residential and commercial landscaping projects. The mechanism of action of atrazine is through inhibition of photosystem II, resulting in the repression of electron transport mechanisms as well as other energy-dependent reactions required for photosynthesis [2,4,5]. Due to the extensive use of this herbicide, atrazine is a routinely detected surface and groundwater contaminant in many Midwestern streams and lakes [6–8].

Organophosphate insecticides are a highly diverse family of organic chemicals generally applied within the same regions of North America as atrazine to control a variety of pests associated with plants, animals, and human health. Although organophosphates (OPs) consist of a broad class of chemical structures, all are acutely toxic and are designed to inhibit the neurotransmitter acetylcholinesterase (AChE), thereby interfering with normal cholinergic nerve transmission. The OPs evaluated in this study, chlorpyrifos, methyl parathion, and diazinon, represent examples of the phosphorothioate class of OP insecticides. This class of OPs is structurally similar and is characterized by thioate structures containing a sulfur moiety attached to a phosphorus center. Oxidative desulfurization by phase I biotransformation enzymes replaces the sulfur atom with an oxygen atom, resulting in an *o*-analog metabolite that is a more effective AChE inhibitor than the parent OP compound.

In the past, several monitoring efforts have determined the co-occurrence of atrazine and OP insecticides in the same urban and agricultural regions of the Midwest. Midwestern agricultural watersheds routinely receive pulses of atrazine during spring and early summer application periods [2]. Although atrazine residues in these low-order watersheds can exceed 40 μg/L during the primary application periods, concentrations of 3 μg/L or less are more typical levels [2,6]. In similar papers, chlorpyrifos and diazinon have been reported to be two of the most frequently detected insecticides in urban and agricultural areas, with detection rates of diazinon exceeding 60% in streams and shallow groundwater in urban areas [9].

Recently, efforts have been made to better understand the phenomena of multiple pesticide interactions and the mechanisms by which these interactions occur. Atrazine is capable of increasing the toxicity of several different insecticides with which the herbicide is combined. For example, atrazine increased parathion (OP insecticide) toxicity in mosquito larvae, *Aedes aegypti*, and fruit flies, *Drosophila melanogaster*, as well as enhanced carbofuran (carbamate insecticide) toxicity in houseflies, *Musca domestica* [10,11]. The results of other studies indicate that atrazine altered the toxic action of insecticides by decreasing mevinphos (phosphate insecticide) and methoxychlor (organochlorine insecticide) toxicity in the midge, *Chironomus tentans* [12]. In contrast, the toxicity of OP insecticides was significantly increased when applied in combination with atrazine using *C. tentans* [13]. The same study also demonstrated that atrazine can accelerate the biotransformation rate of chlorpyrifos to chlorpyrifos *o*-analog in vitro and can increase the amount of chlorpyrifos metabolites

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found in *C. tentans* after in vivo exposure [13]. The combined interaction has been suggested to be the result of cytochrome-dependent monooxygenase induction, resulting in increased biotransformation efficiency of the parent compound to more potent *o*-analog metabolites. Previous studies have supported this hypothesis by demonstrating that the induction of biotransformation enzyme complexes is attributable to atrazine exposure in a variety of invertebrate and vertebrate species [14–16]. This hypothesis was validated by Miota et al. [16]; these authors showed the induction of a 45-kDa protein in atrazine-treated *C. tentans*. The intensity of this atrazine-induced protein is representative of the heme-thiolate membrane-associated proteins within the 45 to 60 kDa molecular-weight enzyme system. This group of proteins function to metabolize a variety of endogenous and exogenous compounds in insects [16].

Atrazine and OPs typically are used in terrestrial environments, but contamination of aquatic habitats with these chemicals is inevitable because of the extensive application of both chemical classes, their moderate solubility in water, and their relatively long environmental half-lives. We used an aquatic amphipod (*Hyalella azteca*) and the common housefly (*M. domestica*) to represent organisms that have the potential to be exposed to atrazine and OP insecticides in Midwestern aquatic and terrestrial habitats. While few studies have examined cytochrome-dependent monooxygenase activity in aquatic invertebrates, studies using *M. domestica* have been conducted to determine specific cytochrome P450 isozyme complexes induced by xenogenous compounds. Therefore, the common housefly may serve as a model in determining the specificity of atrazine toward cytochrome-dependent monooxygenase induction in other invertebrate species.

The current study used a tiered approach to search for atrazine-enhanced toxicity of OPs. For both organisms, the toxicity of individual OPs (chlorpyrifos, methyl parathion, and diazinon) as well as atrazine was determined in the first tier of bioassays. In the second tier, we performed binary mixture experiments by exposing each organism to different atrazine treatments in combination with each OP to evaluate the influence of atrazine on OP toxicity. Acetylcholinesterase activity was assessed in *H. azteca* following exposure to individual and combined treatments of atrazine and each OP in the third tier, while the fourth tier examined the differences in OP toxicity following preexposure of *H. azteca* to atrazine. We also conducted atrazine penetration bioassays to determine whether or not measurable amounts of the herbicide had penetrated the cuticular surface of each organism.

METHODS AND MATERIALS

Organisms

Hyalella azteca were taken from existing colonies maintained by the Environmental Toxicology Core Facility at Wichita State University (Wichita, KS, USA). These aquatic amphipods are cultured in mixed-age chambers according to the standard operating procedures of the U.S. Environmental Protection Agency [17]. Immature amphipods (14--21 d) were collected from the culture chambers and separated from mature and newborn individuals using a No. 18 U.S. standard sieve (1-mm mesh) and a No. 35 U.S. standard sieve (500-μm mesh).

Musca domestica were obtained from a laboratory colony maintained by the Department of Entomology at Kansas State University (Manhattan, KS, USA). Pupae were transferred and reared at the Environmental Toxicology Core Facility at Wichita State University. Individual houseflies, 3 to 5 d old, were used for all bioassays.

Chemicals

Analytical-grade chlorpyrifos was obtained by DowElanco (Indianapolis, IN, USA), while methyl parathion was from Cheminova (Lemuis, Denmark). Atrazine and diazinon were obtained from Chemservice (Westchester, PA, USA). All analytical-grade standards were certified by the suppliers to have a purity >98%. Radiolabeled ^{14}C-atrazine (ring uniformly labeled) was obtained from Sigma (St. Louis, MO, USA) and was determined to have a purity >97% by thin-layer chromatography using a solvent system of benzene:hexane:acetone 50:45:5 v/v/v followed by liquid scintillation (LS) spectroscopy using a Packard 1900 TR scintillation counter (Meriden, CT, USA). The LS spectroscopy was performed using sample counts that were corrected for background and quench using an external standards ratio method [18,19]. The specific activity for ^{14}C-atrazine was 16.6 mCi/mmol. Acetylthiocholine iodide (ATC), bicinchoninic acid solution, Triton X-100, 5,5′-dithio-bis (2-nitrobenzoic acid), and bovine serum albumin also were obtained from Sigma.

Tier I—Acute toxicity bioassays

Hyalella azteca. Acute OP toxicity bioassays were performed for 96 h using 14- to 21-d-old amphipods. Five concentrations of each OP insecticide were used, with each treatment consisting of three replicates with 10 amphipods per replicate. Dosing was performed by making the appropriate dilutions of each chemical in analytical-grade acetone. Test chemicals were delivered to the water using 100 μl of acetone. Solvent controls also were run in conjunction with each toxicity bioassay by introducing the solvent (100 μl acetone) directly into the water. Solvent controls were used to demonstrate that the small volume of acetone added to each beaker did not have an effect on the amphipods. Bioassays were conducted in Precision Scientific® (Grand Rapids, MI, USA) environmental chambers at 20°C with a 16:8-h light:dark photoperiod. To reduce possible adsorption of the pesticides to substrate or food particles, amphipods were not provided a substrate and were not given food during the 96-h test period. In a preliminary study, the lipid content of fed and unfed amphipods did not differ significantly during the 96-h bioassay. Temperature, dissolved oxygen, pH, ammonia, and conductivity in the test media were monitored before and after each bioassay. The endpoint for each bioassay was measured as a lethal concentration (LCXX), representing the concentration where a percentage (XX) of mortality occurred within the test population [20]. Log-probit analysis was used to estimate the LC1, LC5, LC15, and LC50 toxic endpoint concentrations for each of the pesticides.

Musca domestica. Acute OP toxicity bioassays were performed for 24 h using 3- to 5-d-old houseflies. Five concentrations of each OP insecticide were used, with each treatment consisting of three replicates with 10 houseflies per replicate. Pesticides were prepared in acetone and topically applied in 0.5-μl amounts to the ventral abdomen of each housefly using a Hamilton PB600 repeating dispenser (Supelco, Reno, NV, USA). Acetone was used as a carrier to deliver each pesticide due its ability to volatilize rapidly without altering the cuticle of the houseflies [21]. Houseflies were kept in disposable petri dishes (100-mm diam × 15-mm height) lined with moistened filter paper (Whatman, Clifton, NJ, USA). Controls were per-

formed in conjunction with each toxicity bioassay by topically applying the acetone solvent (0.5 μl) alone. Bioassays were conducted in Precision Scientific environmental chambers at 20°C with a 16:8-h light:dark photoperiod. The endpoint for each bioassay was measured as a lethal dose (LDXX), representing the dose causing mortality in a percentage (XX) of the test population. Log-probit analysis was used to estimate the LD1, LD5, LD15, and LD50 toxic endpoint concentrations for each pesticide.

Tier II—Binary toxicity bioassays—Hyalella azteca, Musca domestica

A factorial design was used for both species to assess the combined effect of atrazine and OP insecticides. Individuals were exposed to OP toxic endpoint concentrations consisting of LC1, LC5, LC15, and LC50 and LD1, LD5, LD15, and LD50 values in combination with atrazine treatments of 0, 10, 40, 80, and 200 μg/L for *H. azteca* and 0, 200, and 2,000 ng/mg for *M. domestica*. Toxicant exposure methods and test parameters were the same as those previously described in tier I bioassays.

Statistical comparisons of the treatment classes in this tier were conducted using a two-way analysis of variance (ANOVA) in combination with a Tukey's multiple-comparison test [22]. Percent effect data were arcsine transformed before analysis. The LC50 and LD50 values were estimated for each atrazine and OP treatment using log-probit analysis. Synergistic ratios also were calculated by dividing the LC50 or LD50 value for the control (no atrazine treatment) by the LC50 or LD50 values for each of the atrazine treatments. These ratios were used to determine the magnitude of increase in toxicity existing among treatments.

Tier III—AChE activity bioassays—Hyalella azteca

Determination of AChE activity was conducted according to the method of Ellman et al. [23] as modified by Zhu et al. [24] using ATC as a substrate. The AChE activity was measured using amphipods exposed to individual OPs with and without atrazine treatment. Treatments included a solvent control (acetone), atrazine (200 μg/L), OP (LC1), OP (LC1) + atrazine (200 μg/L), and OP (LC50). Amphipods were exposed to each treatment in the same manner as tier I bioassays, with pesticides being directly introduced into the water using acetone as a carrier. Bioassays were conducted in Precision Scientific environmental chambers at 20°C using a 16:8-h light:dark photoperiod.

After the 96-h exposure period, 10 surviving amphipods were collected from each replicate for each treatment and homogenized with a Potter-Elvehjem tissue homogenizer (Bellco Glass, Vineland, NJ, USA) in ice-cold 0.1 M phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100. We used 175 μl of homogenizing fluid per 10 amphipods. Homogenized amphipods were placed in microcentrifuge tubes and centrifuged at 15,000 *g* for 15 min at 4°C. The supernatants were then collected and transferred to new microcentrifuge tubes. The AChE activity in the supernatants was measured using an enzyme kinetic microplate reader (Molecular Devices, Menlo Park, CA, USA) at a wavelength of 405 nm immediately after 100 μl of the mixture of ATC and 5,5′-dithio-bis (2-nitrobenzoic acid) was added to 50 μl of the supernatant. The final concentrations of ATC and 5,5′-dithio-bis (2-nitrobenzoic acid) in the reaction mixture were 0.25 and 0.40 mM, respectively.

The total protein concentrations in each of the previously prepared AChE activity homogenates were determined using the method of Smith et al. [25]. Total protein was determined by placing 20-μl aliquots of supernatant from each exposure treatment into the wells of a microplate containing 180 μl of a 50:1 volumetric ratio of bicinchoninic acid and 4% (w/v) copper sulfate solution. The microplate was then incubated in a Precision Scientific drying oven at 37°C for 30 min, followed by a cooling period of 5 min at room temperature. Optical density was measured at 560 nm using a microplate reader (Molecular Devices).

The AChE activity for each treatment was compared using the SAS® PROC GLM procedure, with differences determined by a Tukey's multiple-comparison test [22].

Tier IV—Atrazine pretreatment toxicity bioassays—Hyalella azteca

Individuals were pretreated with atrazine concentrations of 0, 40, and 80 μg/L for periods of 48, 96, and 144 h in static-renewal systems before a 96-h OP exposure period. In the 96-h exposures, OP concentrations were at the LC1, LC5, LC15, and LC50 toxic endpoint concentrations, as estimated in tier I bioassays. The OP exposure bioassays were conducted in the same manner as the tier II bioassays (i.e., in a four-by-five factorial design). Due to the increase in bioassay duration, we were concerned with the health of the amphipods if feeding was not included. Therefore, individuals were provided a mixture of yeast, cerophyll, and trout chow (YCT) every 48 h. The amphipods were allowed to feed for 2 h prior to being transferred to freshly treated water.

Statistical applications were the same as those used in tier II bioassays. Percent-effect comparisons of each OP were made with and without atrazine pretreatment, following arcsine transformation, two-way ANOVA, and Tukey's multiple-comparison test [22]. Values representing the LC50 concentrations were estimated for each atrazine treatment class at each pretreatment period using log-probit analysis. Synergistic ratios were used to determine the magnitude of increase in toxicity existing among treatments.

Atrazine penetration bioassays

Hyalella azteca. Bioassays were performed using 14- to 21-d-old amphipods exposed to water amended with ^{14}C-atrazine (13.73 ± 0.14 μg/L) for time periods of 0.5, 1, 6, 24, 48, 72, and 96 h. Bioassays were conducted in Precision Scientific environmental chambers at 20°C with a 16:8-h light:dark photoperiod. Each time period included three replicates with 20 amphipods per replicate. At each sampling time, the amphipods were removed from the treated water and weighed on a Cahn C-33 microbalance (Cerritos, CA, USA). The amphipods were then placed into 20-ml scintillation vials filled with 10 ml of scintillation cocktail (Scinti-Safe Plus 50%, Fisher Scientific, Pittsburgh, PA, USA) and homogenized using a Tekmar Model TM501 Sonic Disruptor (Cincinnati, OH, USA). One-milliliter aliquots of water also were taken from each replicate at each sampling time and placed into scintillation cocktail. Radioactivity was determined using LS spectroscopy. Sample counts were corrected for background and quench using the external standards ratio method [18,19].

Musca domestica. Penetration bioassays were conducted according to the methods of Theisen et al. [26]. Houseflies were individually exposed to ^{14}C-atrazine (1.27 ng/mg) by topical application of the compound to the ventral portion of the abdomen in 0.5-μl amounts. Treated individuals were placed into

Table 1. Toxic endpoint concentrations estimated for *Hyalella azteca* and *Musca domestica*. The values represent a concentration or dose (LCXX or LDXX) causing mortality in a percentage (XX) of the test population, with 95% confidence intervals in parentheses (OP = organophosphate)

Hyalella azteca				
OP (μg/L)	LC1	LC5	LC15	LC50
Chlorpyrifos	0.3×10^{-3} (0.2×10^{-3}–0.3×10^{-3})	1.3×10^{-3} (1.0×10^{-3}–1.5×10^{-3})	4.8×10^{-3} (3.7×10^{-3}–5.5×10^{-3})	42.7×10^{-3} (33.3×10^{-3}–49.2×10^{-3})
Methyl parathion	0.3 (0.2–0.4)	0.5 (0.4–0.8)	0.7 (0.4–1.0)	2.1 (1.0–2.9)
Diazinon	0.9 (0.8–1.2)	1.4 (1.2–1.9)	2.1 (1.8–2.8)	4.3 (3.7–5.6)
Musca domestica				
OP (ng/mg)	LD1	LD5	LD15	LD50
Chlorpyrifos	0.04 (0.02–0.08)	0.1 (0.1–0.2)	0.4 (0.2–0.7)	2.4 (1.2–4.7)
Methyl parathion	0.8 (0.5–1.1)	1.7 (1.0–2.3)	3.2 (1.9–4.3)	9.4 (5.5–12.6)
Diazinon	0.2 (0.1–0.3)	0.6 (0.3–1.0)	1.7 (0.9–2.8)	9.9 (5.1–16.8)

20-ml scintillation vials for periods of 15, 30, 60, 120, 360, 480, and 1,440 min. At each sampling time, houseflies were anesthetized using CO_2 and were placed into a new 20-ml scintillation vials containing 5 ml of acetone for 5 min; this was done to remove the cuticular surface of the insect. Following the cuticle wash, individuals were placed into scintillation cocktail (Scinti-Safe Plus 50%, Fisher Scientific) within a third vial and were homogenized using a Tekmar Model TM501 Sonic Disruptor. Scintillation cocktail (10 ml) was added to the previous two vials as well, and radioactivity was determined in all three vials using LS spectroscopy. Radioactivity was determined for all three vials in order to calculate a mass balance for the system. Sample counts were corrected for background and quench using the external standards ratio method [18,19].

Analysis of chemical concentrations

Pre- and posttest chemical concentrations were monitored to ensure concentration stability throughout the duration of each bioassay (see details below). For *M. domestica* bioassays, pretest samples were taken from each stock solution and quantified by gas chromatography with nitrogen-phosphorus detection. For *H. azteca* bioassays, pre- and posttest concentrations were determined by collecting and extracting treated-water samples before or after each exposure period using solid-phase extraction techniques followed by gas chromatography with nitrogen-phosphorus detection.

Solid-phase extraction was conducted according to the methods of Belden et al. [27], in which solid-phase extraction cartridges (Supelco C_{18}, Bellefonte, PA, USA) were preconditioned with elutions of 3 ml of hexane:acetone (1:1 v/v), 3 ml of methanol, and 3 ml of reagent-grade water. Samples were extracted by vacuum filtering (15 psi, 10–18 ml/min) 250 ml of treated water through the C_{18} cartridge. Analytes bound to the C_{18} column were then eluted using three 3-ml washes of hexane:acetone (1:1 v/v). The extracts were concentrated to 1 ml by evaporation under a stream of ultrapure N_2, after which extracts were analyzed using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a nitrogen-phosphorus detector (220°C, splitless, 0.75-min purge time). The J&W analytical (Folsom, CA, USA) capillary column was a DB-608 (30 m × 0.32 mm with a 0.50-μm film thickness). The oven program was set to start at 100°C for 1 min, increase 7°C/min to 180°C for 2 min, increase 6°C/min to 280°C, and then be held for 4 min. The inlet temperature was 220°C. The carrier (5 ml/min) and make-up (6 ml/min) gas was He. Qualitative identification was based on retention times within 0.50% of standards; quantification was based on peak area using external standards.

The pretest concentrations of all chemicals were within 10% of the initial concentrations. Although posttest concentrations decreased over the 96-h testing period, the drop in concentrations for each chemical never exceeded 15% of the initial concentrations. Therefore, nominal water concentrations for the chemicals were used for all calculations.

RESULTS

Tier I—Acute toxicity bioassays—Hyalella azteca, Musca domestica

Exposure concentrations of the chemicals needed to kill 1, 5, 15, and 50% of the test population of *H. azteca* are shown in Table 1. Exposure doses of the chemicals needed to kill 1, 5, 15, and 50% of the test population of *M. domestica* are also shown. For both organisms, OP toxicity generally was greater for chlorpyrifos, followed by methyl parathion and then diazinon (Table 1). The acute toxicity of atrazine was evaluated for *H. azteca* and *M. domestica* at concentrations up to 10,000 μg/L and 10,000 ng/mg, respectively. The herbicide was not acutely toxic to either organism. Water parameters remained similar in all bioassays. Temperature was maintained at 20 ± 1°C and dissolved oxygen levels were ≥81%. Conductivity remained between 331 to 359 μS/cm and pH ranged from 7.3 to 7.5 during the experiments.

Tier II—Binary mixture toxicity bioassays

Hyalella azteca. Results of tier I testing did not indicate toxicity due to atrazine-only exposure, but atrazine concentrations as low as 40 μg/L significantly increased toxicity of chlorpyrifos to the amphipods. In contrast, atrazine at 80 μg/L significantly increased toxicity of methyl parathion and diazinon to the amphipods. Significant differences among treatment classes were detected by ANOVA for atrazine and chlorpyrifos mixtures, atrazine and methyl parathion mixtures, and

Table 2. Percent-effect comparisons of organophosphate (OP) and atrazine combinations for *Hyalella azteca*. The values are the average percent effect and standard error for that mixture. The totals represent the average and standard error for all samples evaluated within a treatment class. Cell, atrazine, or OP values that are not significantly different are indicated with the same lowercase letter, number, or uppercase letter, respectively. Each row (OP treatment) was considered independently. In all cases, differences were considered to be significant if $p < 0.05$

	Atrazine (μg/L)					
OP (μg/L)	0	10	40	80	200	Total
Chlorpyrifos						
0.3 × 10^{-3}	13.3 (6.7)a	23.3 (3.3)b	33.3 (3.3)b	36.7 (8.8)b	76.7 (6.7)b	36.7 (6.2)A
1.3 × 10^{-3}	26.7 (3.3)a	36.7 (3.3)ab	50.0 (0.0)ab	60.0 (0.0)ab	80.0 (5.8)b	50.6 (5.1)B
4.8 ×	33.3 (3.3)a	43.3 (3.3)ab	50.0 (5.8)ab	63.3 (3.3)b	83.3 (8.8)b	54.7 (5.1)BC
42.7 × 10^{-3}	46.7 (6.7)a	46.7 (3.3)ab	56.7 (3.3)b	70.0 (0.0)b	90.0 (0.0)b	62.0 (4.6)C
Total	30.0 (4.3)1	37.5 (3.0)1,2	47.5 (3.0)2,3	57.5 (4.3)3	82.5 (3.0)4	
Methyl parathion						
0.3	10.0 (0.0)a	13.3 (3.3)a	16.7 (3.3)a	26.7 (3.3)a	30.0 (5.8)a	19.3 (2.5)A
0.5	16.7 (3.3)a	16.7 (6.7)ab	20.0 (0.0)b	30.0 (0.0)b	63.3 (8.8)b	29.3 (5.1)B
0.7	26.7 (3.3)a	26.7 (3.3)b	30.0 (5.8)b	50.0 (5.8)b	90.0 (5.8)b	44.7 (6.8)C
2.1	43.3 (3.3)a	40.0 (5.8)b	43.3 (3.3)b	56.7 (3.3)b	93.3 (6.7)b	55.3 (5.6)D
Total	24.2 (4.0)1	24.2 (3.8)1	27.5 (3.5)1	40.8 (4.2)2	69.2 (8.2)3	
Diazinon						
0.9	3.3 (3.3)a	3.3 (3.3)a	6.7 (3.3)a	20.0 (0.0)a	30.0 (5.8)a	12.7 (3.2)A
1.4	6.7 (3.3)a	6.7 (6.7)ab	6.7 (2.7)b	23.3 (8.8)b	43.3 (3.3)b	17.3 (4.5)AB
2.1	13.3 (8.8)a	13.3 (3.3)a	13.3 (3.3)a	26.7 (6.7)a	46.7 (8.8)a	22.7 (4.3)B
4.3	40.0 (5.8)a	53.3 (3.3)a	50.0 (5.8)a	53.3 (3.3)a	66.7 (3.3)a	52.7 (2.8)C
Total	15.8 (5.0)1	19.2 (6.3)1	19.2 (5.8)1	30.8 (4.7)2	46.7 (4.7)2	

atrazine and diazinon mixtures (Table 2). The *p*-values in each case were highly significant ($p < 0.0001$). Tukey's multiple comparison test showed that OP toxicity increased as atrazine concentrations increased ($p < 0.05$).

In this tier, the LC50 concentrations estimated for individual OPs and OPs in combination with each atrazine treatment were used to calculate synergistic ratios. Atrazine treatments of 200 μg/L appeared to have a large effect on chlorpyrifos, methyl parathion, and diazinon, with 2.8-, 2.9-, and 3.0-fold increases in toxicity, respectively (Table 3). This is a substantial increase in toxicity when considering that levels of 50 times this amount of atrazine did not cause toxicity by itself.

Musca domestica. Atrazine did not significantly influence the toxicity of chlorpyrifos, methyl parathion, or diazinon in the houseflies, based on two-way ANOVA and Tukey's multiple comparison test (Table 4). Due to the nonsignificant interaction for *M. domestica* and the tiered design of this study, tiers III and IV used only *H. azteca*.

Tier III—AChE inhibition bioassays—Hyalella azteca

Exposure to each OP in combination with atrazine (200 μg/L) significantly inhibited AChE activity compared with corresponding OP-only treatments (Fig. 1). Significant reductions in AChE activity did not occur in response to atrazine-only exposures (200 μg/L) relative to the solvent controls (Fig. 1). Chlorpyrifos-only treatments at the LC1 (0.30 ng/L) level had a 41% reduction in AChE activity compared with solvent controls, while the LC1 level of chlorpyrifos in combination with atrazine (200 μg/L) reduced AChE activity by 61%. Methyl parathion (0.31 μg/L) in combination with atrazine (200 μg/L) reduced AChE activity by 49% compared with solvent controls; we observed a 12% reduction in AChE at the LC1 methyl parathion-only treatment (Fig. 1). Diazinon treatments resulted in effects similar to those noted for methyl parathion and chlorpyrifos. The AChE was 27% lower in LC1-only (0.90 μg/L) treatments compared with solvent controls and 43% lower in the diazinon and atrazine (200 μg/L) combination (Fig. 1).

Table 3. Summary of LC50 values (μg/L) estimated for *Hyalella azteca* for each organophosphate insecticide (OP) at the level of atrazine exposure indicated[a]

	Atrazine (μg/L)				
OP (μg/L)	0	10	40	80	200
Chlorpyrifos	0.04	0.03	0.03	0.02	0.02
SR	—	1.0	1.6	2.0	2.8
Methyl parathion	2.1	2.0	1.8	1.2	0.7
SR	—	1.0	1.0	1.7	2.9
Diazinon	4.3	3.6	3.5	2.2	1.5
SR	—	1.0	1.0	2.0	3.0

[a] SR = synergistic ratio calculated by $SR = LC50_{control}/LC50_{treatment}$. The SR values were not calculated if no significant difference ($p < 0.05$) in the atrazine treatment was found compared with the control (no atrazine treatment) in the two-way analysis of variance and Tukey's multiple-comparison test.

Tier IV—Atrazine pretreatment toxicity bioassays—Hyalella azteca

Pretreatment to atrazine concentrations for 48 and 96 h did not significantly affect the toxicities of the OP chemicals, although the toxicity of each OP was increased when the amphipods were pretreated with atrazine for 144 h before OP-only exposure (Table 5). The ANOVA results showed that the differences among the 144-h pretreatment classes were significant for atrazine (40 μg/L) and chlorpyrifos mixtures, atrazine (80 μg/L) and methyl parathion mixtures, and atrazine (80 μg/L) and diazinon mixtures (Table 5). The *p*-values in each case were highly significant ($p \leq 0.02$). Results obtained from a Tukey's multiple comparison test indicate a significant increase in OP toxicity when the amphipods are pretreated with atrazine at 40 and 80 μg/L for 144 h (Table 5).

Synergistic ratios were calculated by dividing the LC50 of the control (no atrazine) by the LC50 value for each atrazine treatment. These values were used as a measure of the magnitude of toxicity increase that exists among treatments. Atrazine (40 μg/L) pretreatments for 144 h caused a 1.8-fold increase in chlorpyrifos toxicity, while methyl parathion and diazinon became 1.2 and 1.4 times more toxic, respectively, after pretreatment with atrazine (80 μg/L) for 144 h.

Table 4. Percent-effect comparisons of organophosphate (OP) and atrazine combinations for *Musca domestica*. The values are the average percent effect and standard error for that mixture. The totals represent the average and standard error for all samples evaluated within a treatment class. Cell, atrazine, or OP values that are not significantly different are indicated with the same lowercase letter, number, or uppercase letter, respectively. Each row (OP treatment) was considered independently. In all cases, differences were considered to be significant if $p < 0.05$

	Atrazine (ng/mg)			
OP (ng/mg)	0	200	2000	Total
Chlorpyrifos				
0.04	26.7 (3.3)a	36.7 (3.3)b	23.3 (3.3)a	28.9 (2.6)A
0.1	33.3 (3.3)a	36.7 (6.7)a	30.0 (0.0)a	33.3 (2.4)AB
0.4	36.7 (6.7)a	40.0 (0.0)a	53.3 (3.3)b	43.3 (3.3)B
2.4	83.3 (6.7)a	73.3 (3.3)a	73.3 (3.3)a	76.7 (2.9)C
Total	45.0 (7.1)1	46.7 (5.0)1	45.0 (6.1)1	
Methyl parathion				
0.8	46.7 (3.3)b	40.0 (0.0)a	53.3 (6.7)a	46.7 (2.9)A
1.7	53.3 (3.3)b	43.3 (3.3)a	43.3 (3.3)a	47.8 (2.2)A
3.2	70.0 (0.0)b	56.7 (3.3)a	76.7 (3.3)c	67.8 (3.2)B
9.4	90.0 (0.0)a	93.3 (3.3)a	93.3 (6.7)a	92.2 (2.2)C
Total	65.0 (5.2)1	59.2 (6.3)1	66.7 (6.3)1	
Diazinon				
0.2	33.3 (3.3)a	30.0 (0.0)a	26.7 (3.3)a	30.0 (1.7)A
0.6	33.3 (6.7)a	33.3 (3.3)a	40.0 (5.8)a	35.6 (2.9)A
1.7	63.3 (3.3)a	60.0 (5.8)a	63.3 (6.7)a	62.2 (2.8)B
9.9	86.7 (3.3)a	83.3 (6.7)a	80.0 (5.8)a	83.3 (2.9)C
Total	54.2 (7.0)1	51.7 (6.8)1	52.5 (6.6)1	

Atrazine penetration bioassays

Hyalella azteca. The movement of ^{14}C-atrazine into *H. azteca* is shown in Figure 2A. Atrazine concentrations in the water during the 96-h exposure period were 15.60 ± 0.75 μg/L. In the first 0.5 h of exposure, 5.66 ± 0.41 ng/mg of atrazine was detected in the organisms, while 17.62 ± 1.14 ng/mg of atrazine was found in the amphipods after 48 h. After 96 h of exposure, the amphipods contained 21.19 ± 2.27 ng/mg of the compound.

Musca domestica. Atrazine concentrations recovered from *M. domestica* after 1,440 min of exposure were examined to reveal the movement of atrazine through the insect's system (Fig. 2B). The total recovery of atrazine in the 1,440-min exposure period was 87.2 ± 4.6%. Atrazine bound to the cuticular surface of the houseflies appeared to decrease over time, with an initial concentration of 0.87 ± 0.06 ng/mg in the first 15 min and a final concentration of 0.02 ± 0.00 ng/mg after 1,440 min of exposure. Homogenate extracts indicated an increase in atrazine concentrations in the first 120 min of exposure, with 0.51 ± 0.04 ng/mg being the highest amount recovered. After 120 min of exposure, atrazine concentrations begin to decrease in the homogenates. We found a final concentration of 0.09 ± 0.01 ng/mg at 1,440 min. Atrazine eliminated from the houseflies increased over time, with an initial concentration of 0.07 ± 0.01 ng/mg and a final concentration of 0.70 ± 0.06 ng/mg.

DISCUSSION

In our study, atrazine (40 μg/L for chlorpyrifos or 80 μg/L for methyl parathion and diazinon) in combination with chlorpyrifos, methyl parathion, or diazinon resulted in 1.6-, 1.7-, and 2.0-fold increases in toxicity, respectively, to *H. azteca*. Furthermore, the toxicity of each OP increased by a factor of approximately three when the atrazine concentration was increased to 200 μg/L (Table 3). Tier III bioassays with *H. azteca* showed greater AChE inhibition (20–40%) in individuals exposed to atrazine and OP (LC1) combinations compared with individuals exposed to OP-only treatments (LC1) (Fig. 1). These findings suggest that atrazine increased the biotransformation efficiency of chlorpyrifos, methyl parathion, and diazinon, resulting in a greater production of *o*-analog metabolites, which in turn reduced AChE activity and increased OP insecticide toxicity.

The results of studies by other investigators strengthen the argument that an indirect mechanism is responsible for increasing the toxicity of insecticides to atrazine-treated organisms. For example, Kao et al. [15] reported an induction of cytochrome P450 isozymes in southern armyworm larvae (*Spodoptera eridania*) given an atrazine-contaminated diet, while similar studies have shown that atrazine can induce cytochrome P450 isozymes in rainbow trout (*Oncorhynchus mykiss*) and the cabbage moth (*Mamestra brassica*) [14]. Miota et al. [16] also demonstrated a cytochrome P450-mediated aldrin epoxidation in *C. tentans* that had been pretreated with atrazine. By comparing aldrin epoxidase activity measurements in control and atrazine-exposed midges, they found a 45-kDa protein of increased intensity in atrazine-treated midges [16]. As previously stated, the molecular weight of this protein is similar to the weights of heme-thiolate membrane-associated proteins (45–60 kDa) having enzyme functions associated with the metabolism of various endogenous and exogenous compounds in many insects [16]. The induction of cytochrome P450 isozymes could increase the biotransformation efficiency of OP parent compounds to *o*-analog metabolites, which are more effective AChE inhibitors. An increase in *o*-analog formation, in turn, would allow for greater AChE inhibition, resulting in an increase in toxicity, as observed by Belden and Lydy [13] using *C. tentans* and reported here using *H. azteca*.

Previous efforts have focused on the toxic interaction between atrazine and insecticide mixtures. Our study is unique in that we found that the toxicity of OP insecticides appears to be significantly affected in organisms in contact with atrazine before exposure to an OP. In tier IV bioassays, *H. azteca* preexposed to atrazine concentrations for 144 h were more sensitive to OP toxicity, although not to the same extent as when both chemicals were dosed simultaneously (Tables 2 and 5). Interestingly, this observation suggests that an organism

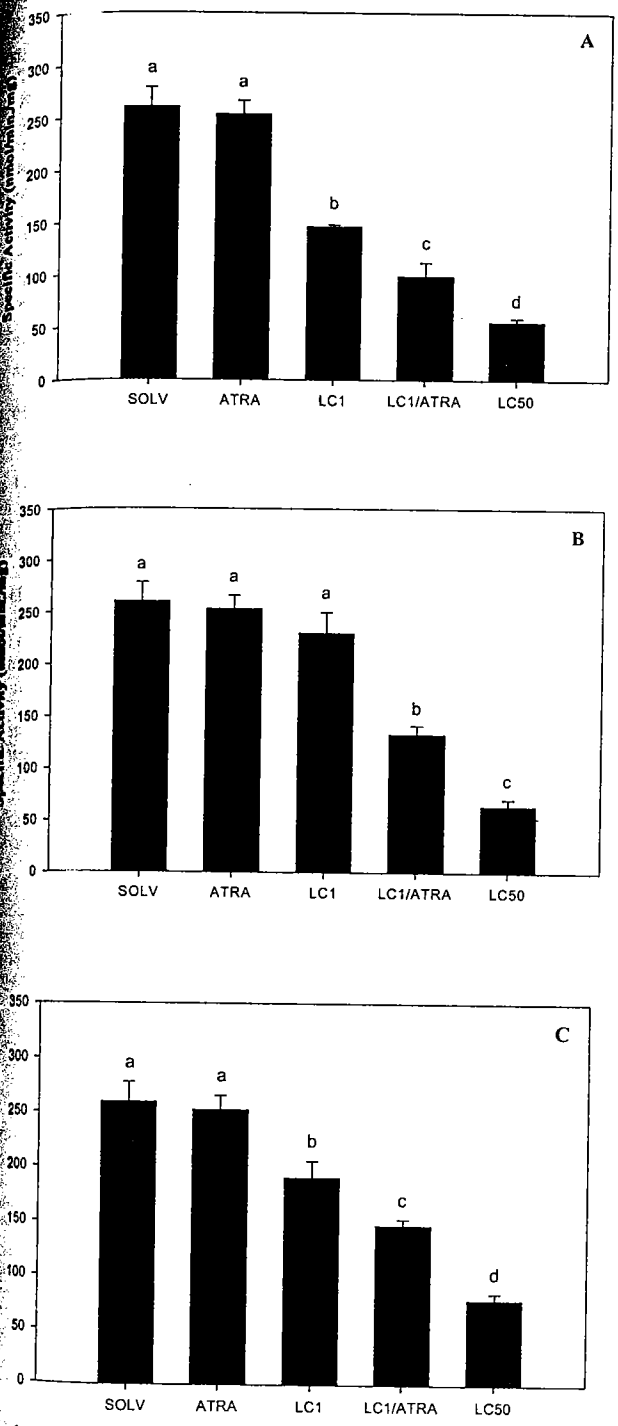

Fig. 1. Comparison of specific activities of acetylcholinesterase in *Hyalella azteca* exposed to organophosphate (OP) and atrazine treatments. All bars represents the mean ± 1 standard error (n = 6) of enzyme activities (nmol/min/mg protein). Significant differences in specific activity are indicated by different letters (Tukey's multiple comparison $p < 0.05$). (**A**) Chlorpyrifos treatments: LC1 and LC50 concentrations were 3.0×10^{-4} and 4.3×10^{-2} µg/L, respectively. (**B**) Methyl parathion treatments: LC1 and LC50 concentrations were 0.31 and 2.08 µg/L, respectively. (**C**) Diazinon treatments: LC1 and LC50 concentrations were 0.90 and 4.29 µg/L, respectively. Atrazine (ATRA) treatments were 200 µg/L, while the solvent (SOLV) treatments contained 100 µl acetone.

Table 5. Percent-effect comparisons of organophosphates (OP) following atrazine pretreatment for *Hyalella azteca*. The values are the total average percent effect and standard error for all samples evaluated within a treatment class. Significant differences in the percent effect for each compound following atrazine pretreatment are indicated by different letters. Each row (OP treatment) was considered independently. In all cases, differences were considered to be significant if $p < 0.05$

	Atrazine pretreatment duration (h)		
OP	48	96	144
Chlorpyrifos			
0 µg/L atrazine	28.3 (10.8)a	12.5 (5.4)a	11.7 (4.9)a
40 µg/L atrazine	26.7 (10.6)a	18.3 (5.8)a	21.7 (6.4)b
Methyl parathion			
0 µg/L atrazine	25.0 (8.4)a	13.3 (4.8)a	12.5 (4.5)a
80 µg/L atrazine	30.0 (9.7)a	20.0 (6.7)a	23.3 (5.6)b
Diazinon			
0 µg/L atrazine	17.5 (4.8)a	7.5 (2.5)a	8.3 (3.0)a
80 µg/L atrazine	21.7 (6.0)a	15.0 (2.9)b	18.3 (4.4)b

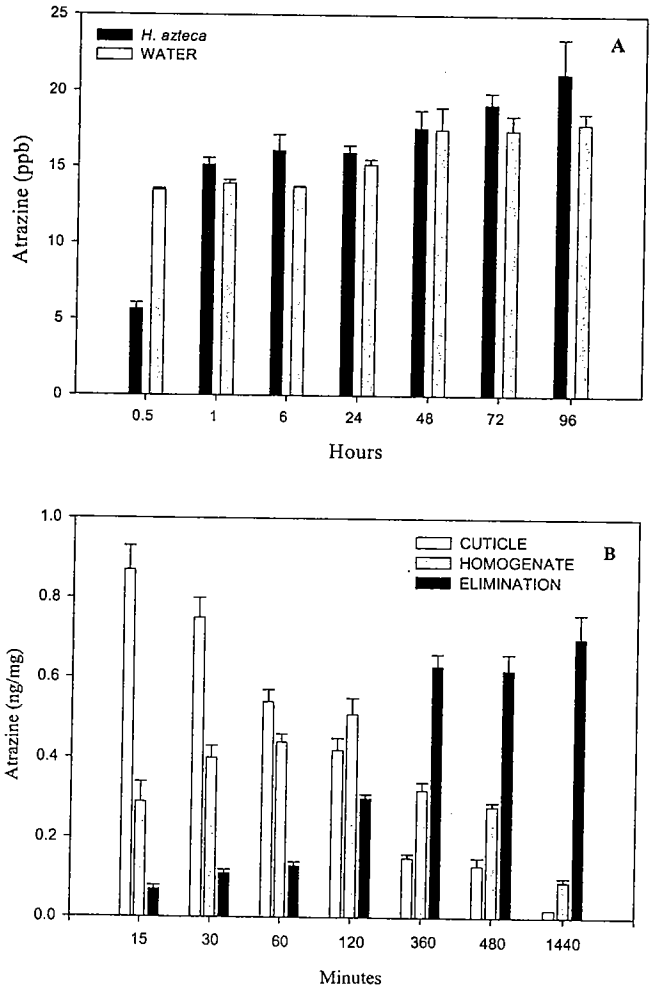

Fig. 2. Concentrations of atrazine entering *Hyalella azteca* and *Musca domestica*. (**A**) Each bar represents the mean ± 1 standard error (n = 3) of atrazine concentrations in *H. azteca* (ng/mg) and the water (ng/ml). (**B**) Each series represents a phase in which atrazine passes through *M. domestica*. Open bars represent the cuticle, gray bars represent homogenate, and black bars represent elimination. Each bar represents the mean ± 1 standard error (n = 3) of atrazine concentrations (ng/mg). Atrazine was applied at a concentration of 1.3 ng/mg. The total average recovery for each time point was 87.2 ± 4.6%.

may need to be exposed to atrazine only for a period of time long enough to allow induction of the cytochrome P450 isozymes needed to increase OP biotransformation. Greater vulnerability of the organism to the OP is then the result.

Bioassays with *M. domestica* did not show enhanced sensitivity to the OPs due to atrazine exposure. Acute toxicity was evident for each OP individually in tier I bioassays (Table 1); we speculated that the nonsignificant interaction between atrazine and OP insecticides occurred because atrazine was unable to penetrate the housefly cuticle. The results from the penetration bioassays, however, indicated that measurable amounts of atrazine penetrated the insect's cuticle where the herbicide could be readily absorbed into the system and eventually eliminated (Fig. 2B). Similar to the housefly bioassays, *H. azteca* showed a significant accumulation of atrazine in the system over a 96-h period (Fig. 2A). The influence of atrazine on insecticide toxicity may be strongly species specific as well as compound specific. Other reports have shown that an insect's ability to degrade xenobiotic compounds involves an enzyme complex equipped with a recognition system that, when stimulated by an appropriate exogenous substrate, can produce enzymes capable of degrading those compounds [28]. Based on this observation, the nonsignificant interaction of atrazine and OP insecticides reported for *M. domestica* is presumed to be the result of atrazine being unable to induce the enzyme complex capable of biotransforming OP insecticides or that detoxification isozymes are also being activated that minimize the effect [13,16].

In summary, the efforts of this study have added to the growing body of research investigating atrazine-influenced toxicity. These results illustrate a significant interaction between two pesticide classes known to commonly co-occur in the environment. Although atrazine significantly affected OP toxicity in *H. azteca*, it did not influence OP toxicity in *M. domestica*. This discrepancy justifies the need for studies of atrazine-influenced toxicity on other organisms to strengthen the evaluation of risk of these chemical mixtures. The results of this study and those investigations referred in this article pertain to the acute effects of atrazine-influenced toxicity only. The effects of other triazine-class herbicides on insecticide toxicity should be assessed as well as the examination of atrazine-influenced toxicity in organisms chronically exposed to insecticides.

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SECTION 3

ANNOTATED PAGES FROM PEER REVIEWERS' PROFILE DOCUMENTS

ANNOTATED PAGES FROM

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SUMMARY

Atrazine, deethylatrazine, simazine, diazinon, and nitrate were chosen as the subject mixture for this interaction profile because they frequently occur together in rural well water. The exposures of greatest concern for this scenario are intermediate and chronic oral exposures. No pertinent health effects data or physiologically based pharmacokinetic (PBPK) models were located for the complete mixture. Therefore, the exposure-based screening assessment of potential health hazards for this mixture depends on an evaluation of the health effects data for the individual components, and on the joint toxic action and mechanistic data for various combinations of the components. This profile discusses and evaluates the evidence for joint toxic action among atrazine, deethylatrazine, simazine, diazinon, and nitrate, and recommends how to incorporate concerns regarding possible interactions or additivity into public health assessments of people who may be exposed to mixtures of these chemicals.

Effects of concern for this mixture include reproductive effects (atrazine, deethylatrazine, and simazine), neurological effects (diazinon), and hematological effects (nitrate). Although none of the components has been classified as a carcinogen, atrazine and simazine can react with nitrite (nitrate metabolite) ~~in the~~ environment and *in vivo* to form N-nitrosoatrazine and N-nitrososimazine. Structure-activity considerations raise a concern for potential carcinogenicity of these nitrosamines.

The following recommendations are appropriate when hazard quotients (ratio of exposure to health guidance value) for two or more of the mixture components equal or exceed 0.1. To screen this mixture for potential reproductive health hazard, an endpoint-specific hazard index for reproductive effects should be estimated for atrazine, deethylatrazine, and simazine (triazines). The weight-of-evidence (WOE) analysis for interactions among these components indicates high confidence in the additivity assumption, which is the basis for the hazard index. The potential effect of diazinon and nitrate on the reproductive toxicity of these triazines is uncertain. Separate hazard quotients are recommended for the neurotoxicity of diazinon and the hematological toxicity of nitrate. The WOE analysis indicates that because the triazine components may potentiate the neurologic toxicity of diazinon, the hazard quotient for diazinon may tend to underestimate the hazard. Confidence in these predictions is medium to low. No information regarding the impact of interactions on the hematological toxicity of nitrate was available, so uncertainty regarding the impact of the other components on this effect of nitrate is high. Chemical interactions of atrazine and simazine with nitrite can result in the formation of N-nitroso-atrazine and N-nitrososimazine, which are suspected to have carcinogenic potential, and which are more genotoxic than the parent triazine compounds. Thus, the WOE analysis predicts an increase in

route of exposure for this mixture is likely to be oral and the durations of concern are intermediate and chronic.

Before evaluating the relevance of joint toxic action data for these chemicals, some understanding of endpoints of concern for oral exposure to this mixture is needed. The endpoints of concern include the critical effects that are the bases for MRLs or other health guidance values, and any other endpoints that may become significant because they are shared targets of toxicity or due to interactions (ATSDR 2001a).

In order to satisfy the requirements of the Food Quality Protection Act (FQPA) to assess the cumulative effects of chemicals that have a common mechanism of toxicity, certain triazines pesticides, including atrazine, its metabolite deethylatrazine (also known as desethylatrazine, desethyl s-triazine), and simazine, are being reevaluated by the Environmental Protection Agency's (EPA) (2002c) Office of Pesticide Programs. The EPA (2002c) has concluded that these triazines should be considered as a *Common Mechanism Group* based on suppression of the luteinizing hormone ovulatory surge and the resulting effects on reproductive function and reproductive development. EPA (2002b) has derived a new chronic reference dose (RfD) for atrazine and its chlorinated metabolites, including deethylatrazine, based on reproductive effects; this RfD is not on the Integrated Risk Information System (IRIS) (2003), but its derivation includes a consideration of mechanistic and toxicological data that have become available since the RfD on IRIS was derived. EPA has not yet derived a new RfD for simazine. Further explanation is provided in Appendices A and B. ATSDR (2003) is evaluating atrazine in a new toxicological profile that is a post-public comment draft as of this writing. ATSDR (2003) did not derive intermediate and chronic oral MRLs because the lowest-observed-adverse-effect level (LOAEL) for both durations was lower than applicable no-observed-adverse-effect levels (NOAELs), and was considered a serious LOAEL for reproductive effects (including anestrus). Thus, reproductive effects are the effects of concern for atrazine, deethylatrazine, and simazine.

Diazinon's critical effect, which is the basis of ATSDR (1996) MRLs and EPA (2000; IRIS 2003) RfDs, is neurological, due to inhibition of acetylcholinesterase, primarily by its activated metabolite diazoxon. Nitrate, through reduction to nitrite, causes methemoglobinemia, which is the critical effect for EPA's (IRIS 2003) RfD.

None of these chemicals has been classified as a carcinogen (see Appendices), but a chemical interaction between atrazine and nitrite and between simazine and nitrite results in the formation of N-nitroso-atrazine and N-nitrososimazine. These nitrosamines have not been adequately tested for carcinogenicity, but structure-activity considerations raise the suspicion that they may have carcinogenic potential.

Thus, the endpoints of concern for this mixture are reproductive, neurological, hematological, and carcinogenic. The structures and the Chemical Abstracts Service (CAS) Registry Numbers of these chemicals are provided in Appendix E.

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 (EC_{50}) 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). When tested at the levels of EPA maximum contaminant levels (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 pesticides 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* TA 1535, 1537, 1538, 98, or 100 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 pesticides, 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, and in the female Sprague-Dawley rat, results in mammary gland tumors. This 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). These mechanistic considerations are discussed in more detail 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 larvae reported that environmentally ~~relevant concentrations of atrazine~~ potentiated the acute neurotoxicity (measured as the inability of midges to perform normal swimming motions) of diazinon in 96-hour static toxicity tests (Belden and Lydy 2000). Atrazine was not acutely toxic to midges even at the limit of water solubility, 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 organophosphorous pesticides. This conclusion is based on induction of the metabolism of another organophosphorous pesticide, chlorpyrifos, by atrazine in additional experiments in this study.

Diazinon is metabolically activated by cytochrome P450 to diazoxon, which binds to acetylcholin-esterase, 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 study in midges 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 nitrosamines from pesticide amino groups and nitrite is of concern because many nitrosamines are carcinogenic. 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 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 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

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

BINWOE: >IIICb (+1 x 0.32 x 0.32 x 0.79 = +0.08)

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 a potentially more toxic compound, N-nitrosoatrazine.

Mechanistic Understanding - Atrazine and nitrite 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). In addition, 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 relatively clear, but the mechanism of action of this compound is unknown. Therefore, a rating of III is selected 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. The clastogenicity of N-nitrosoatrazine in cultured human lyphocytes 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. Neither atrazine nor N-nitrosoatrazine caused mutations in *S. typhimurium* with or without rat liver S9 (Ishidate 1983; Ishidate et al. 1981). These results indicate that the chemical interaction of atrazine with nitrite to form N-nitrosoatrazine may be a greater-than-additive interaction for genotoxicity. The relevance to other toxicological effects including cancer, however, is less clear, and, given the lack of supporting data, results in a rating of C for toxicological significance.

Modifying Factors - A modifying factor for *in vitro* data (b) is appropriate.

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

BINWOE: >IIICb (+1 x 0.32 x 0.32 x 0.79 = +0.08)

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 a potentially more toxic compound, N-nitrososimazine.

Mechanistic Understanding - Simazine and nitrite were shown to react at acidic pH to form N-nitroso-simazine (Eisenbrand et al. 1975b). The formation of N-nitrosoatrazine from simazine and nitrite also has been detected in rats following oral administration (Dmitrenko et al. 1996). Thus, there is some evidence of a chemical interaction resulting in the formation of N-nitrososimazine, but the mechanism of action of this compound is unknown. Therefore, a rating of III is selected 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). 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). The relevance to other toxicological effects including cancer, however, is unclear, and, given the lack of actual joint toxic action data, results in a rating of C for toxicological significance.

Modifying Factors - A modifying factor for *in vitro* data (b) is appropriate.

Additional Uncertainties - Confidence in this assessment is lower than that for atrazine and nitrate.

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 relevant to potential carcinogenicity of N-nitrosoatrazine and N-nitroso-simazine, chemical interaction products of atrazine and simazine with nitrate.

3. Recommendation for Exposure-Based Assessment of Joint Toxic Action of the Mixture

As discussed in the introduction, the mixture of atrazine, deethylatrazine, simazine, diazinon, and nitrate was chosen as the subject for this interaction profile on the basis of an analysis of the most frequently occurring mixtures in rural domestic and public water-supply wells (Squillace et al. 2002). The exposure scenario of greatest concern for this mixture is intermediate to chronic exposure low-level oral exposure.

No adequate epidemiological or toxicological studies and no PBPK models are available for this mixture. Recommendations for exposure-based screening for the potential health hazard of this mixture are based on ATSDR (2001a) guidance, and comprise a components-based approach. This approach is used for the components with hazard quotients that equal or exceed 0.1, when at least two of the mixture components fulfill this criterion. Hazard quotients are the ratios of exposure estimates to noncancer health guidance values, such as MRLs. If only one or if none of the mixture components has a hazard quotient of this magnitude, no further assessment of the joint toxic action is needed because additivity and/or interactions are unlikely to result in significant health hazard. As discussed by ATSDR (1992, 2001a), the exposure-based assessment of potential health hazard is a screening approach, to be used in conjunction with biomedical judgment, community-specific health outcome data, and community health concerns to assess the degree of public health hazard.

Because there are sensitive reproductive endpoints in common to the triazine components of the mixture, the recommended approach (ATSDR 2001a) for atrazine/deethylatrazine and simazine is to estimate an endpoint-specific hazard index (by summing the hazard quotients for these components) for reproductive effects, using the guidance values shown in Table 6, or newer values as they become available. Hazard quotients are the ratios of exposures to MRLs, target-organ toxicity doses (TTDs), or other health guidance values. This process is shown in the following equation:

$$HI_{REPRO} = \frac{(E_{Atr}+E_{DEA})}{TTD_{Atr/DEA\ REPRO}} + \frac{E_{Smz}}{TTD_{Smz\ REPRO}}$$

where HI_{REPRO} is the hazard index for reproductive toxicity, E_{Atr} is the exposure to atrazine (as the oral intake in mg/kg/day), E_{DEA} is the exposure to deethylatrazine (as the oral intake in mg/kg/day), and $TTD_{Atr/DEA\ REPRO}$ is the TTD (in mg/kg/day) for the reproductive effects of oral exposure to atrazine and

deethylatrazine. Similarly, E_{Smz} is the exposure to simazine (as oral intake in mg/k/day) and $TTD_{Smz\ REPRO}$ is the TTD for the reproductive effects of oral exposure to simazine.

The weight-of-evidence analysis for interactions, summarized in the BINWOE determinations in Table 7, indicates that additivity is an appropriate assumption for the reproductive effects of atrazine/deethyl-atrazine and simazine, which act by a common mode of action on these endpoints, and can be considered dose additive. Confidence in the additivity assumption is high. The influence of diazinon and nitrate on the reproductive toxicity of these triazines, however, is indeterminate.

The neurological effects of diazinon are to be assessed with a separate hazard quotient for this chemical, because they are unique to diazinon. This hazard quotient may underestimate the potential hazard of diazinon during co-exposure to atrazine, deethylatrazine, and simazine because the BINWOEs for the effects of these components on diazinon predict a greater-than-additive interaction (in this case, potentiation), but confidence in these predictions is medium to low. The influence of nitrate is indeterminate.

The hematological effects of nitrate also are to be assessed with a separate hazard quotient. The influence of the other mixture components on nitrate's hematological toxicity are indeterminate.

If the hazard index for reproductive effects exceeds one, it provides preliminary evidence that the mixture may constitute a health hazard due to the joint toxic action of components on that endpoint (ATSDR 2001a). Similar preliminary conclusions apply if the hazard quotient for diazinon's neurological effects or the hazard quotient for nitrate's hematological effects exceeds one. The prediction that the triazines may potentiate the neurological toxicity of diazinon increases the concern. When these screening criteria are exceeded, additional evaluation is needed using biomedical judgment, and taking into consideration community-specific health outcome data, and community health concerns to assess the degree of public health hazard (ATSDR 1992, 2001a).

The potential carcinogenicity of the complete mixture is unknown. None of the individual components have been classified as carcinogenic (see Appendices), but atrazine and simazine can react with nitrite, the metabolite of nitrate, to form N-nitrosoatrazine and N-nitrososimazine. The potential carcinogenicity of these nitrosamines has not been investigated adequately. Genotoxicity studies indicate they are more

genotoxic than the triazines from which they were formed. Confidence in greater-than-additive predictions for carcinogenicity, however, are low, as reflected in the BINWOEs.

Table 6. MRLs and TTDs for Intermediate and Chronic Oral Exposure to Chemicals of Concern
(See Appendices A, B, C, and D for Details)

Endpoint	Chemical			
	Atrazine/ deethylatrazine (mg/kg/day)	Simazine (mg/kg/day)	Diazinon (mg/kg/day)	Nitrate (mg/kg/day)
Reproductive	$1.8x10^{-3}$ [a]	$1.8x10^{-3}$ [a]	NA	NA
Neurological	NA	NA	$2x10^{-4}$ [b]	NA
Hematological	NA	NA	NA	1.6[c]

[a]Chronic dietary population adjusted dose (PAD) for atrazine and its chlorinated metabolites (EPA 2002b), adopted as target-organ toxicity dose (TTD) for atrazine and deethylatrazine (combined), and as an interim TTD for simazine.
[b]Intermediate oral MRL for diazinon.
[c]Chronic oral RfD for nitrate, adopted as TTD.

NA = not applicable

Table 7. Matrix of BINWOE Determinations for Intermediate or Chronic Simultaneous Oral Exposure to Chemicals of Concern

		ON TOXICITY OF			
		Atrazine/ deethylatrazine	Simazine	Diazinon	Nitrate
EFFECT OF	Atrazine/ deethylatrazine		=IA (0) r	>IIC (+0.32) n	? (0) h >IIICb (+0.08) c
	Simazine	=IA (0) r		>IIIC (+0.10) n	? (0) h >IIICb (+0.08) c
	Diazinon	? (0) r	? (0) r		? (0) h
	Nitrate	? (0) r >IIICb (+0.08) c	?(0) r >IIICb (+0.08) c	? (0) n	

r = reproductive, n = neurological, h = hematological, c = carcinogenic

The BINWOE determinations were explained in Section 2.3. No pertinent interactions data were available for the pairs of chemicals classified as indeterminate (?), and mechanistic information appeared inadequate, so indeterminate ratings were assigned to these pairs.

BINWOE scheme (with numerical weights in parentheses) condensed from ATSDR (2001a, 2001b):

DIRECTION: = additive (0); > greater than additive (+1): < less than additive (–1); ? indeterminate (0)

MECHANISTIC UNDERSTANDING:
I: direct and unambiguous mechanistic data to support direction of interaction (1.0);
II: mechanistic data on related compounds to infer mechanism(s) and likely direction (0.71);
III: mechanistic data do not clearly indicate direction of interaction (0.32).

TOXICOLOGIC SIGNIFICANCE:
A: direct demonstration of direction of interaction with toxicologically relevant endpoint (1.0);
B: toxicologic significance of interaction is inferred or has been demonstrated for related chemicals (0.71);
C: toxicologic significance of interaction is unclear (0.32).

MODIFYING FACTORS:
1: anticipated exposure duration and sequence (1.0);
2: different exposure duration or sequence (0.79);
a: *in vivo* data (1.0);
b: *in vitro* data (0.79);
i: anticipated route of exposure (1.0);
ii: different route of exposure (0.79).

4. Conclusions

A component-based approach is recommended for the exposure-based screening assessment of potential hazards to public health from exposure to this mixture. The recommendations include the estimation of a hazard index for the reproductive effects of the triazine components of this mixture: atrazine/deethyl-atrazine and simazine. In addition, separate hazard quotients are to be estimated for the neurological effects of diazinon and the hematological effects of nitrate. This approach is appropriate when the hazard quotients of at least two of the components equal or exceed 0.1 (ATSDR 2001a). The WOE evaluation of interactions indicates high confidence in the additivity assumption (hazard index) for atrazine/deethylatrazine and simazine, and uncertainty regarding the potential effect of the other mixture components on the reproductive toxicity of these triazines. Further conclusions from the WOE analysis are that the triazine components may potentiate the neurological toxicity of diazinon such that the hazard quotient may underestimate the degree of hazard, but confidence in that conclusion is medium to low. No information regarding the impact of interactions on the hematological toxicity of nitrate was available, so uncertainty is high for this endpoint. Although the individual components of the mixture have not been classified as carcinogens, the triazine components may interact with nitrate (as the nitrite metabolite) to form N-nitrosoatrazine and N-nitrososimazine, which are suspected to have carcinogenic potential, and which are more genotoxic than the parent triazine compounds. Uncertainty regarding this prediction is high because of the lack of adequate data on these nitrosamines.

The mechanism of action with regard to pancreatic toxicity in dogs and guinea pigs appears to be inhibition of butyrylcholinesterase in the pancreas and its smooth muscle sphincters, leading to ductal hypertension and cholinergic hyperstimulation of the acinar cells (Dressel et al. 1980; Frick et al. 1987).

C.4 Health Guidelines

ATSDR (1996) derived an intermediate inhalation MRL of 0.009 mg/m^3 for brain acetylcholinesterase inhibition diazinon based on a NOAEL of 0.46 mg/m^3 in a 21-day study in rats. An uncertainty factor of 30 was applied. The LOAEL (20% decrease in brain acetylcholinesterase) was 1.57 mg/m^3.

ATSDR (1996) derived an intermediate oral MRL of 2x10^{-4} mg/kg/day based on a NOAEL of 0.021 mg/kg/day for brain acetylcholinesterase inhibition in dogs given diazinon in their food daily for 13 weeks. An uncertainty factor of 100 was used. The LOAEL (31% decrease in erythrocyte and brain acetylcholinesterase) was 5.9 mg/kg/day.

EPA (IRIS 2003) does not have an online file for diazinon.

The EPA (2000) Office of Pesticide Programs derived acute and chronic RfDs of 2.5x10^{-3} and 2x10^{-4} mg/kg/day based on NOAELs for cholinesterase inhibition of 2.5 mg/kg/day (in rats) and 0.02 mg/kg/day in seven feeding studies (in rats and dogs), respectively. No additional FQPA safety factor was needed; the PADs are therefore the same as the RfDs.

NTP (2003) and IARC (2003) do not include diazinon in their listings. The EPA (2000) Office of Pesticide Programs classified diazinon as a *not likely human carcinogen* based on the lack of evidence of carcinogenicity in mice and rats.

C.5 Derivation of Target-Organ Toxicity Dose (TTD) Values

TTDs were not derived. The intermediate oral MRL based on neurological effects is appropriate for use as a chronic guidance value as well, and is the same as the chronic oral RfD developed by EPA (2000).

Appendix D: Background Information for Nitrate

Nitrate occurs naturally in foods, particularly in vegetables. Inorganic fertilizers, livestock waste, and septic tank discharges are primary contributors to nitrate contamination of drinking water (NRC 1995). The structures of nitrate and its metabolite nitrite are shown in Appendix E.

D.1 Toxicokinetics

Available studies indicate that oral absorption of nitrate is nearly 100% (for reviews, see EPA 1990 and WHO 1978). Witter (1979, cited in EPA 1990) administered oral radioactive nitrate ion to two male volunteers; one received the nitrate 1 hour after a large meal, the other about 10 hours after eating. In the subject who had recently eaten, the radioactivity had a disappearance half-life from the stomach of about 30 minutes, but the radioactivity in the pylorus remained constant, suggesting that the nitrate had moved to the small intestine rather than being absorbed through the stomach. In the second subject, the disappearance half-life was 10 minutes. Studies in animals have also demonstrated that the bulk of an orally-administered nitrate exposure is absorbed through the small intestine, likely through the upper portion of that organ. Absorbed nitrate is distributed throughout the body, but does not appear to accumulate in any organ (EPA 1990).

The major metabolic pathway for nitrate is conversion to nitrite, and then to ammonia. Small amounts of nitrate, perhaps 5–10% of the total exposure, are converted to nitrite by bacteria in the saliva, stomach, and small intestine. This reaction is pH dependent, with no nitrate reduction occurring below pH 4 and above pH 9, and the presence of oxygen inhibits the reduction of nitrite to ammonia. Absorbed nitrite rapidly reacts with hemoglobin in the blood to form methemoglobin, which in adults, is rapidly converted to oxyhemoglobin, then back to hemoglobin. In infants, particularly those under 3 months old, these reducing systems are not fully developed, which may result in a buildup of methemoglobin in the blood. Due to the higher stomach pH typically found in infants, it is believed that they also convert more nitrate to nitrite in the stomach than adults. There are large species differences in the rate of reaction of nitrite with hemoglobin, paralleled by similar differences in the rates of reduction of methemoglobin, making extrapolation of results from animal data to humans problematic. Another potential metabolic pathway, though less prevalent than the reaction with hemoglobin, is the reaction of nitrite with endogenous molecules to form N-nitroso compounds, many of which have toxic effects, including carcinogenicity.

stomach. The stomach of adults is typically too acidic to allow for significant bacterial growth and the resulting conversion of nitrate to nitrite. Additionally, the enzymes involved in the conversion of methemoglobin to hemoglobin do not fully develop in humans until between 3 and 6 months after birth, resulting in an increased susceptibility to methemoglobinemia.

As mentioned in Section D.1, the reaction rates for the nitrite-hemoglobin reaction vary considerably across species (many animal species lack nitrate-reducing bacteria), as do the rates of the reactions reducing methemoglobin back to functional hemoglobin. In addition, since the rates of conversion of nitrate to nitrite by bacteria can vary within individuals, the extent of nitrate toxicity can vary greatly depending on age and other factors within both humans and animals.

D.4 Health Guidelines

ATSDR has not published a toxicological profile for nitrates. No MRL values are available.

EPA (IRIS 2003) has derived an oral RfD of 1.6 mg/kg/day for nitrate, based on a NOAEL of 1.6 mg/kg/day for methemoglobinemia in exposed infants (Bosch et al. 1950; Walton 1951). An uncertainty factor of 1 was applied to the NOAEL since the study was performed in a sensitive population of humans (infants age 0–3 months). No reference concentration (RfC) for nitrate has been derived, and nitrate has not undergone an evaluation of carcinogenic potential by EPA.

D.5 Derivation of Target Organ Toxicity Dose (TTD) Values

In the absence of a toxicological profile and MRLs for nitrate, the chronic oral RfD of 1.6 mg/kg/day for nitrate (IRIS 2003) can be adopted as the TTD for hematological effects.

TTD_{HEMATO} = 1.6 mg/kg/day

D.6 References

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SUMMARY

Atrazine, deethylatrazine, simazine, diazinon, and nitrate were chosen as the subject mixture for this interaction profile because they frequently occur together in rural well water. The exposures of greatest concern for this scenario are intermediate and chronic oral exposures. No pertinent health effects data or physiologically based pharmacokinetic (PBPK) models were located for the complete mixture. Therefore, the exposure-based screening assessment of potential health hazards for this mixture depends on an evaluation of the health effects data for the individual components, and on the joint toxic action and mechanistic data for various combinations of the components. This profile discusses and evaluates the evidence for joint toxic action among atrazine, deethylatrazine, simazine, diazinon, and nitrate, and recommends how to incorporate concerns regarding possible interactions or additivity into public health assessments of people who may be exposed to mixtures of these chemicals.

Effects of concern for this mixture include reproductive effects (atrazine, deethylatrazine, and simazine), neurological effects (diazinon), and hematological effects (nitrate). Although none of the components has been classified as a carcinogen, atrazine and simazine can react with nitrite (nitrate metabolite) in the environment and *in vivo* to form N-nitrosoatrazine and N-nitrososimazine. Structure-activity considerations raise a concern for potential carcinogenicity of these nitrosamines.

The following recommendations are appropriate when hazard quotients (ratio of exposure to health guidance value) for two or more of the mixture components equal or exceed 0.1. To screen this mixture for potential reproductive health hazard, an endpoint-specific hazard index for reproductive effects should be estimated for atrazine, deethylatrazine, and simazine (triazines). The weight-of-evidence (WOE) analysis for interactions among these components indicates high confidence in the additivity assumption, which is the basis for the hazard index. The potential effect of diazinon and nitrate on the reproductive toxicity of these triazines is uncertain. Separate hazard quotients are recommended for the neurotoxicity of diazinon and the hematological toxicity of nitrate. The WOE analysis indicates that because the triazine components may potentiate the neurologic toxicity of diazinon, the hazard quotient for diazinon may tend to underestimate the hazard. Confidence in these predictions is medium to low. No information regarding the impact of interactions on the hematological toxicity of nitrate was available, so uncertainty regarding the impact of the other components on this effect of nitrate is high. Chemical interactions of atrazine and simazine with nitrite can result in the formation of N-nitroso-atrazine and N-nitrososimazine, which are suspected to have carcinogenic potential, and which are more genotoxic than the parent triazine compounds. Thus, the WOE analysis predicts an increase in

1. Introduction

The primary purpose of this Interaction Profile for atrazine, deethylatrazine, diazinon, nitrate, and simazine is to evaluate data on the toxicology of the "whole" mixture and the joint toxic action of the chemicals in the mixture in order to recommend approaches for assessing the potential hazard of this mixture to public health. To this end, the profile evaluates the whole mixture data (if available), focusing on the identification of health effects of concern, adequacy of the data as the basis for a mixture Minimal Risk Level (MRL), and adequacy and relevance of physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) models for the mixture. The profile also evaluates the evidence for joint toxic action—additivity and interactions—among the mixture components. A weight-of-evidence (WOE) approach is commonly used in these profiles to evaluate the influence of interactions in the overall toxicity of the mixture. The weight-of-evidence evaluations are qualitative in nature, although the Agency for Toxic Substances and Disease Registry (ATSDR) recognizes that observations of toxicological interactions depend greatly on exposure doses and that some interactions appear to have thresholds. Thus, the interactions are evaluated in a qualitative manner to provide a sense of what influence the interactions may have when they do occur. The profile provides environmental health scientists with ATSDR Division of Toxicology's (DT) recommended approaches for the incorporation of the whole mixture data or the concerns for additivity and interactions into an assessment of the potential hazard of this mixture to public health. These approaches can then be used with specific exposure data from hazardous waste sites or other exposure scenarios.

The atrazine, deethylatrazine, diazinon, nitrate, and simazine mixture was chosen as the subject for this interaction profile based on analyses of frequently occurring mixtures in groundwater. As part of the National Water-Quality Assessment Program of the U.S. Geological Survey, untreated groundwater samples were collected from 1,255 domestic (rural) wells and 242 public water-supply wells (Squillace et al. 2002), and analyzed for 60 volatile organic compounds (VOCs), 83 pesticides, and nitrate. The most frequently occurring four-chemical mixture in these groundwater samples consisted of two triazine herbicides and a metabolite (atrazine, simazine, and deethylatrazine), plus nitrate. [Of the 144 monitored chemicals, nitrate was the one that most frequently exceeded a drinking water standard or health criteria.] Atrazine and simazine did not exceed drinking water criteria. Diazinon was the most frequently detected organophosphate pesticide, and exceeded the drinking water health advisory in one well. The primary

it would be helpful to know what the nitrate standard was.

route of exposure for this mixture is likely to be oral and the durations of concern are intermediate and chronic.

Before evaluating the relevance of joint toxic action data for these chemicals, some understanding of endpoints of concern for oral exposure to this mixture is needed. The endpoints of concern include the critical effects that are the bases for MRLs or other health guidance values, and any other endpoints that may become significant because they are shared targets of toxicity or due to interactions (ATSDR 2001a).

In order to satisfy the requirements of the Food Quality Protection Act (FQPA) to assess the cumulative effects of chemicals that have a common mechanism of toxicity, certain triazines pesticides, including atrazine, its metabolite deethylatrazine (also known as desethylatrazine, desethyl s-triazine), and simazine, are being reevaluated by the Environmental Protection Agency's (EPA) (2002c) Office of Pesticide Programs. The EPA (2002c) has concluded that these triazines should be considered as a *Common Mechanism Group* based on suppression of the luteinizing hormone ovulatory surge and the resulting effects on reproductive function and reproductive development. EPA (2002b) has derived a new chronic reference dose (RfD) for atrazine and its chlorinated metabolites, including deethylatrazine, based on reproductive effects; this RfD is not on the Integrated Risk Information System (IRIS) (2003), but its derivation includes a consideration of mechanistic and toxicological data that have become available since the RfD on IRIS was derived. EPA has not yet derived a new RfD for simazine. Further explanation is provided in Appendices A and B. ATSDR (2003) is evaluating atrazine in a new toxicological profile that is a post-public comment draft as of this writing. ATSDR (2003) did not derive intermediate and chronic oral MRLs because the lowest-observed-adverse-effect level (LOAEL) for both durations was lower than applicable no-observed-adverse-effect levels (NOAELs), and was considered a serious LOAEL for reproductive effects (including anestrus). Thus, reproductive effects are the effects of concern for atrazine, deethylatrazine, and simazine.

Diazinon's critical effect, which is the basis of ATSDR (1996) MRLs and EPA (2000; IRIS 2003) RfDs, is neurological, due to inhibition of acetylcholinesterase, primarily by its activated metabolite diazoxon. Nitrate, through reduction to nitrite, causes methemoglobinemia, which is the critical effect for EPA's (IRIS 2003) RfD.

excellent review for the interested reader.

"better if suspicion replaced by scientific issue."

None of these chemicals has been classified as a carcinogen (see Appendices), but a chemical interaction between atrazine and nitrite and between simazine and nitrite results in the formation of N-nitroso-atrazine and N-nitrososimazine. These nitrosamines have not been adequately tested for carcinogenicity, but structure-activity considerations raise the suspicion that they may have carcinogenic potential.

Thus, the endpoints of concern for this mixture are reproductive, neurological, hematological, and carcinogenic. The structures and the Chemical Abstracts Service (CAS) Registry Numbers of these chemicals are provided in Appendix E.

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 physiologically based pharmacokinetic (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, 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 $F_{2\alpha}$ (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 $F_{2\alpha}$ 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 (EC_{50}) 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). When tested at the levels of EPA maximum contaminant levels (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 pesticides 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.

good review to support conclusion.

Neither atrazine nor simazine nor the mixture of the two produced a mutagenic response in *Salmonella typhimurium* TA 1535, 1537, 1538, 98, or 100 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 pesticides, 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, and in the female Sprague-Dawley rat, results in mammary gland tumors. This 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). These mechanistic considerations are discussed in more detail 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).

good review for the interested reader.

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 larvae reported that environmentally relevant concentrations of atrazine potentiated the acute neurotoxicity (measured as the inability of midges to perform normal swimming motions) of diazinon in 96-hour static toxicity tests (Belden and Lydy 2000). Atrazine was not acutely toxic to midges even at the limit of water solubility, 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 organophosphorous pesticides. This conclusion is based on induction of the metabolism of another organophosphorous pesticide, chlorpyrifos, by atrazine in additional experiments in this study.

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

discuss absence of olfactory effect such as seen with Atrazine and Simazine?

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. 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, does not provide information regarding mode of joint toxic action, and is not supported by other studies of atrazine or nitrate (see Appendix A).

It was my impression that this study raised doubts about nitrosamines carcinogenicity in humans, regardless of the shortcomings

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 NO_3-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

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). Neither atrazine nor N-nitrosoatrazine was mutagenic in *Salmonella typhimurium* TA98, TA100, or TA1537 with or without rat liver S9 (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, but neither chemical causes gene mutations. 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 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) by 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 faction.

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 nitrosamines from pesticide amino groups and nitrite is of concern because many nitrosamines are carcinogenic. Simazine and nitrite were shown to react at acidic pH to form N-nitrososimazine (Eisenbrand et al. 1975b).

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

BINWOE: =IA (0 x 1 x 1 = 0)

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 pesticides, including atrazine and simazine, and their chlorinated metabolites, including deethylatrazine, 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 neuro-endocrine 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 $F_{2\alpha}$ (Moore and Lower 2001). The only other studies of joint toxic action showed no evidence of synergism regarding clastogenicity in Chinese hamster ovary cells (Taets et al. 1998), no mutagenic effects of either chemical or the mixture on *Salmonella typhimurium* (Eisenbeis et al. 1981), and concentration addition with regard to inhibition of reproduction of cultures of *Chorella fusca* (green algae) (Faust et al. 1993). The toxicological relevance of results in algae is questionable. The genotoxicity studies do 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. The mechanistic understanding indicates a common mechanism of toxicity for atrazine, deethyl-atrazine, and simazine, and therefore strongly supports the prediction of additivity. Therefore, a rating of A is chosen for toxicological significance.

The species difference therefore decreases toxic potential. Is this included in the BINWOE?

I

B?

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

BINWOE: >IIC (+1 x 0.71 x 0.32 = +0.23)

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, and induction by diazinon of metabolic activation of another organophosphorous pesticide (Belden and Lydy 2000), and similar mechanism of neurotoxicity in insects and humans.

Mechanistic Understanding - Diazinon is metabolically activated by cytochrome P450 to diazoxon, which binds to acetylcholinesterase, inhibiting its ability to hydrolyze acetylcholine. This results in continued neurological stimulation. This mechanism of action applies to both insects and mammals. Atrazine induced the metabolism of another organophosphorous pesticide, chlorpyrifos, and potentiated its acute neurotoxicity to midges (Belden and Lydy 2000). A similar mechanism can be inferred for atrazine's potentiation of the acute neurotoxicity of diazinon to midges in the same study. Because of uncertainties inherent in extrapolating from insects to humans, and because the mechanism is inferred from a similar chemical, a rating of II is chosen for mechanistic understanding.

Toxicological Significance - Atrazine potentiated the acute neurotoxicity (inability of midges to perform normal swimming motions) of diazinon in 96-hour static toxicity tests (Belden and Lydy 2000). Organophosphorous pesticides 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 some relevance to humans. Because there is only one relevant study, because of concerns regarding potential differences between insects and humans, and because the mechanistic basis is inferred from a different organophosphorous pesticide, confidence is low and a rating of C is appropriate.

Why isn't rating of B appropriate?

B?

3. Recommendation for Exposure-Based Assessment of Joint Toxic Action of the Mixture

As discussed in the introduction, the mixture of atrazine, deethylatrazine, simazine, diazinon, and nitrate was chosen as the subject for this interaction profile on the basis of an analysis of the most frequently occurring mixtures in rural domestic and public water-supply wells (Squillace et al. 2002). The exposure scenario of greatest concern for this mixture is intermediate to chronic exposure low-level oral exposure.

No adequate epidemiological or toxicological studies and no PBPK models are available for this mixture. Recommendations for exposure-based screening for the potential health hazard of this mixture are based on ATSDR (2001a) guidance, and comprise a components-based approach. This approach is used for the components with hazard quotients that equal or exceed 0.1, when at least two of the mixture components fulfill this criterion. Hazard quotients are the ratios of exposure estimates to noncancer health guidance values, such as MRLs. If only one or if none of the mixture components has a hazard quotient of this magnitude, no further assessment of the joint toxic action is needed because additivity and/or interactions are unlikely to result in significant health hazard. As discussed by ATSDR (1992, 2001a), the exposure-based assessment of potential health hazard is a screening approach, to be used in conjunction with biomedical judgment, community-specific health outcome data, and community health concerns to assess the degree of public health hazard.

Because there are sensitive reproductive endpoints in common to the triazine components of the mixture, the recommended approach (ATSDR 2001a) for atrazine/deethylatrazine and simazine is to estimate an endpoint-specific hazard index (by summing the hazard quotients for these components) for reproductive effects, using the guidance values shown in Table 6, or newer values as they become available. Hazard quotients are the ratios of exposures to MRLs, target-organ toxicity doses (TTDs), or other health guidance values. This process is shown in the following equation:

$$HI_{REPRO} = \frac{(E_{Atr}+E_{DEA})}{TTD_{Atr/DEA\ REPRO}} + \frac{E_{Smz}}{TTD_{Smz\ REPRO}}$$

where HI_{REPRO} is the hazard index for reproductive toxicity, E_{Atr} is the exposure to atrazine (as the oral intake in mg/kg/day), E_{DEA} is the exposure to deethylatrazine (as the oral intake in mg/kg/day), and $TTD_{Atr/DEA\ REPRO}$ is the TTD (in mg/kg/day) for the reproductive effects of oral exposure to atrazine and

deethylatrazine. Similarly, E_{Smz} is the exposure to simazine (as oral intake in mg/k/day) and $TTD_{Smz\ REPRO}$ is the TTD for the reproductive effects of oral exposure to simazine.

The weight-of-evidence analysis for interactions, summarized in the BINWOE determinations in Table 7, indicates that additivity is an appropriate assumption for the reproductive effects of atrazine/deethyl-atrazine and simazine, which act by a common mode of action on these endpoints, and can be considered dose additive. Confidence in the additivity assumption is high. The influence of diazinon and nitrate on the reproductive toxicity of these triazines, however, is indeterminate.

as well as other organophosphates.

The neurological effects of diazinon are to be assessed with a separate hazard quotient for this chemical, because they are unique to diazinon. This hazard quotient may underestimate the potential hazard of diazinon during co-exposure to atrazine, deethylatrazine, and simazine because the BINWOEs for the effects of these components on diazinon predict a greater-than-additive interaction (in this case, potentiation), but confidence in these predictions is medium to low. The influence of nitrate is indeterminate.

The hematological effects of nitrate also are to be assessed with a separate hazard quotient. The influence of the other mixture components on nitrate's hematological toxicity are indeterminate.

Does such data exist. If not, this should be stated

If the hazard index for reproductive effects exceeds one, it provides preliminary evidence that the mixture may constitute a health hazard due to the joint toxic action of components on that endpoint (ATSDR 2001a). Similar preliminary conclusions apply if the hazard quotient for diazinon's neurological effects or the hazard quotient for nitrate's hematological effects exceeds one. The prediction that the triazines may potentiate the neurological toxicity of diazinon increases the concern. When these screening criteria are exceeded, additional evaluation is needed using biomedical judgment, and taking into consideration community-specific health outcome data, and community health concerns to assess the degree of public health hazard (ATSDR 1992, 2001a).

The potential carcinogenicity of the complete mixture is unknown. None of the individual components have been classified as carcinogenic (see Appendices), but atrazine and simazine can react with nitrite, the metabolite of nitrate, to form N-nitrosoatrazine and N-nitrososimazine. The potential carcinogenicity of these nitrosamines has not been investigated adequately. Genotoxicity studies indicate they are more

revealed that, after controlling for potential confounding factors including maternal smoking, atrazine was more strongly correlated with intrauterine growth retardation than were the other herbicides, but the herbicides (atrazine, cyanazine, metolachlor) were intercorrelated. In addition, estimates of exposure and confounding factors were made on the community rather than individual level.

Atrazine causes neuroendocrine, reproductive, and reproductive developmental effects in experimental animals. Animal studies have shown that atrazine disrupts estrus cyclicity (i.e., irregular ovarian cycling and changes in the number and/or percentage of days in estrus and diestrus) and alters plasma hormone levels in rats and pigs. These effects appear to be mediated by changes in the hypothalamic-pituitary-ovary axis that are species-, and even strain-, specific. In 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, and persistent estrus results. In other strains of rats, atrazine causes elevated progesterone levels, which leads to pseudo-pregnancy and persistent diestrus (ATSDR 2003).

The mechanism of reproductive senescence in humans does not involve disruption of hormonal regulation, but is initiated by depletion of ova in the ovaries, which ultimately results in decreased plasma estrogen levels. Therefore, disruption of the menstrual cycle or acceleration of reproductive senescence is not anticipated to occur in humans as a result of atrazine exposure. However, it is not known whether atrazine will cause other perturbations in the hypothalamus-pituitary-gonad axis resulting in reproductive effects in human (ATSDR 2003).

Developmental effects have been observed following pregestational, gestational, and lactational oral exposure of rat and rabbit dams and peripubertal oral exposure of rats to atrazine. The observed effects included impaired development of the reproductive system, postimplantation losses, decreases in fetal body weight, incomplete ossification, and neurodevelopmental effects (ATSDR 2003).

A number of epidemiology studies have investigated the carcinogenic potential of atrazine or triazine herbicides (ATSDR 2003; IARC 1999a). These studies include cohort studies of triazine manufacturing workers, case-control studies of farmers using atrazine or triazines, and ecological studies of populations in agricultural areas with high atrazine or triazine use and populations of areas with atrazine-contaminated drinking water. Results of these studies were inconclusive. Odds ratios, standardized

mortality ratios (SMRs), or relative risks generally were not elevated or were not statistically significantly elevated after adjustment for exposure to other pesticides. A few studies reported statistically significant correlations or elevated odds ratios for cancer of the prostate (Mills 1998), breast (Kettles et al. 1997), ovary (Donna et al. 1989), or stomach (Van Leeuwen et al. 1999) and triazine or atrazine exposure. These studies, however, had no individual measures of exposure and/or no accounting for exposure to other pesticides, and are not confirmed by the other available epidemiological studies on the same chemicals.

Good!

Statistically significant earlier onset or increased incidences of mammary tumors were observed in female Sprague-Dawley rats, but not in female F344 rats or in mice (ATSDR 2003). The early onset of mammary tumors in female Sprague-Dawley rats is believed to be the result of atrazine-induced acceleration of reproductive senescence, as further explained under mechanisms of action.

Deethylatrazine has not been explicitly considered in the epidemiology studies. Because it is frequently detected in surface and groundwaters that contain atrazine (Gilliom et al. 1999; Squillace et al. 2002), studies that involved exposure to atrazine or triazines through drinking water probably included exposure to deethylatrazine.

Relative Dose?

A few studies of deethylatrazine have been performed in animals. In these studies, deethylatrazine generally produced the same effects as atrazine. Diaminochlorotriazine, a metabolite of both atrazine and deethylatrazine, has been tested more extensively and caused similar reproductive function and reproductive developmental effects, and carcinogenic effects (mammary gland tumors in Sprague-Dawley female rats) affects as did atrazine (EPA 2002c). Therefore, it is reasonable to assume that deethylatrazine will do so as well.

A.3 Mechanisms of Action

The primary target of atrazine in some animal species is the female reproductive system. Altered estrus cyclicity has been observed in Sprague-Dawley, Long-Evans, and Donryu rats following exposure to ≥5 mg/kg/day atrazine for intermediate or chronic durations and to a single dose of 300 mg/kg/day. Atrazine does not appear to have estrogenic activity. Atrazine is thought to disrupt endocrine function, and the estrus cycle, primarily through its action on the central nervous system in a manner very similar to the known mechanism of reproductive senescence in some strains of rats. In certain strains of rats,

including Sprague-Dawley and Long-Evans, reproductive senescence begins by 1 year of age, and results from inadequate stimulation of the pituitary by the hypothalamus to release LH; low serum levels of LH leads to anovulation, persistent high plasma levels of estrogen, and persistent estrus. Atrazine apparently accelerates the process of reproductive senescence in these strains of rats (ATSDR 2003).

Atrazine has been shown to induce mammary tumor formation in female Sprague-Dawley rats, but not male Sprague-Dawley or male or female F344 rats. This effect is also thought to be the result of acceleration of reproductive senescence, as described above. Both the failure to ovulate and the state of persistent estrus lead to constant elevated serum levels of endogenous estrogen, which may result in tumor formation in estrogen-sensitive tissues. The rat does not appear to be an adequate model for potential atrazine carcinogenicity in women because reproductive senescence in women involves ovarian depletion and decreased serum estrogen levels instead of decreasing hypothalamic function and increased serum estrogen levels (ATSDR 2003; EPA 2002a, 2002b, 2002c).

Relative dose?

As previously stated, atrazine has been shown to alter serum LH and prolactin levels in Sprague-Dawley rats by altering the hypothalamic control of these hormones (Cooper et al. 2000). LH and prolactin are released from the pituitary in response to GnRH from the hypothalamus. One proposed mechanism is that atrazine decreases the hypothalamic secretion of norepinephrine, which in turn decreases the release of GnRH (EPA 2002a, 2002c). Another proposed mechanism is that atrazine disrupts hypothalamic release of GnRH by interfering with the binding of some ligands, but not others, to the $GABA_A$ receptors in a noncompetitive manner (ATSDR 2003).

A.4 Health Guidelines

ATSDR (2003) did not derive inhalation MRLs for atrazine because of the lack of suitable data.

ATSDR (2003) derived an acute oral MRL of 0.01 mg/kg/day based on a NOAEL of 1 mg/kg/day for decreased body weight gain in rabbits administered atrazine by gavage on gestation days 7–19, and using an uncertainty factor of 100. The LOAEL was 5 mg/kg/day; slight but statistically significant reductions in food consumption and body weight gain were seen at this dose level.

ATSDR (2003) did not derive intermediate or chronic oral MRLs because anestrus, a serious effect, occurred at the lowest LOAEL.

EPA derived an oral RfD of 0.035 mg/kg/day based on a NOAEL of 3.5 mg/kg/day in a chronic dietary study in rats, and using an uncertainty factor of 100 (IRIS 2003). The LOAEL was 25 mg/kg/day. The critical effects were decreased body weight gain in the rat study and cardiac toxicity in a 1-year dietary study in dogs. This RfD was verified by EPA in 1993; significant new studies have been published since that time (IRIS 2003).

Do you want to reference FQPA reasoning for child safety factor?

The EPA (2002b) Office of Pesticide Programs derived an acute RfD of 0.10 mg/kg/day based on a weight-of-evidence analysis of four developmental studies. EPA (2002b) derived a chronic RfD of 0.018 mg/kg/day based on attenuation of the LH surge and estrus cycle disruptions in female Sprague Dawley rats. A FQPA safety factor of 10 was applied to protect infants and children when assessing dietary (food + drinking water) exposures, resulting in a acute population adjusted dose (PAD) of 0.01 mg/kg/day and a chronic PAD of 0.0018 mg/kg/day. These RfDs and PADs are for atrazine and its chlorinated metabolites (including deethylatrazine), which are considered to have equivalent toxicity to atrazine.

The EPA (2002c) Office of Pesticide Programs has concluded that atrazine, deethylatrazine, diamino-chlorotriazine, deisopropylatrazine, simazine, and propazine should be considered a *Common Mechanism Group* for cumulative risk assessment due to their ability to suppress the pituitary LH surge resulting in effects on reproductive function and reproductive development.

NTP (2003) does not include atrazine in its listings.

IARC (1999a) classified atrazine as *not classifiable as to its carcinogenicity to humans* (Group 3) based on inadequate evidence in humans and sufficient evidence in experimental animals.

EPA has not published a cancer assessment of atrazine on IRIS (2003). The EPA (2002a, 2002b) Office of Pesticide Programs classified atrazine and its chlorinated metabolites (including deethylatrazine) as *not likely to be carcinogenic to humans.*

B.2 Health Effects

Some of the epidemiological studies reviewed in Appendix A were on agricultural exposure to trazines in Midwestern states, and did not specify whether exposure to simazine occurred. Because atrazine and cyanazine are the main triazines used as herbicides in the corn belt of the Midwest, it is likely that exposures were mainly to atrazine and cyanazine (Snedeker and Clark 1998). The Ontario farm survey studies reviewed in Appendix A listed atrazine and cyanazine, but not simazine. IARC (1999b) stated that no human reproductive and developmental effects data were available for simazine, and no human cancer data were available for simazine alone.

Relative dose?

Studies in rats indicate that simazine has effects on reproductive function and reproductive development similar to those of atrazine, as do its metabolites deisopropylatrazine and diaminochlorotriazine (EPA 2002c). Also, simazine and diaminochlorotriazine cause mammary gland tumors in Sprague-Dawley female rats (EPA 2002c).

B.3 Mechanisms of Action

The mechanism of action of simazine and its metabolites deisopropylatrazine and diaminochlorotriazine is considered to be the same as for atrazine as described in Section A.3 with regard to neuroendocrine, reproductive, and carcinogenic effects (EPA 2002c).

B.4 Health Guidelines

ATSDR has not developed a toxicological profile or MRLs for simazine.

EPA derived an oral RfD of 0.005 mg/kg/day based on a NOAEL of 0.52 mg/kg/day in a chronic dietary study in rats, and using an uncertainty factor of 100 (IRIS 2003). The LOAEL was 5.3 mg/kg/day. The critical effects were reduction in weight gain and hematological changes (mainly depression of red cell parameters. This RfD was verified by EPA in 1993; significant new studies have been published since that time (IRIS 2003).

The EPA (2002c) Office of Pesticide Programs has concluded that atrazine, deethylatrazine, diamino-chlorotriazine, deisopropylatrazine, simazine, and propazine should be considered a *Common Mechanism*

Appendix C: Background Information for Diazinon

Diazinon is an organophosphorous insecticide. The structure of diazinon and its toxic metabolite, diazoxon, are provided in Appendix E.

C.1 Toxicokinetics

Diazinon is rapidly absorbed from the gastrointestinal tract, based on case reports of ingestion of diazinon formulation or solution, on single oral dose studies in rats and dogs, and on repeated oral dose studies in rats. Absorption in rats and dogs was at least 85% of the dose (ATSDR 1996; WHO 1998). The main features of diazinon metabolism are:

- activation of diazinon through conversion of the P=S moiety to P=O, resulting in the toxic intermediate, diazoxon;
- cleavage of the ester bonds of diazinon and diazoxon resulting in 2-isopropyl-4-methyl-6-hydropyrimidine (from both), diethylphosphorothioc acid (from diazinon), and diethylphosphoric acid (from diazoxon);
- oxidation of the isopropyl substituent of 2-isopropyl-4-methyl-6-hydropyrimidine to the corresponding primary and tertiary alcohols;
- glutathione-mediated cleavage of the ester bond with the formation of a glutathione conjugate (minor pathway).

The resulting metabolites are excreted primarily in the urine (ATSDR 1996; WHO 1998).

The metabolic activation of diazinon is carried out by microsomal cytochrome P450 monooxygenases. A single study of diazinon in rat hepatic microsomes has reported that CYP2B1/2 are the major P450 isozymes that catalyze the production of diazoxon (Fabrizi et al. 1999).

C.2 Health Effects

The principal toxic effect of diazinon in humans, experimental animals, and insects is acetylcholinesterase inhibition. Acetylcholine is a neurotransmitter in the central and peripheral neurons. Inhibition of

acetylcholinesterase, the enzyme that breaks down and terminates the action of acetylcholine, results in the accumulation of acetylcholine at acetylcholine receptors leading to continued stimulation.

In humans and experimental animals, the accumulation of acetylcholine results in cholinergic responses in the peripheral (muscarinic and nicotinic) and central nervous system and neuromuscular junctions. These cholinergic responses, seen in severe acetylcholinesterase inhibition, include excessive glandular secretions (salivation, lacrimation, rhinitis), miosis, bronchoconstriction, vasodilation, hypotension, diarrhea, nausea, vomiting, urinary incontinence, and bradycardia associated with muscarinic receptor stimulation. Tachycardia, mydriasis (dilation of the pupil), muscle fasciculations, cramping, twitching, muscle weakness, and muscle paralysis are associated with nicotinic receptor stimulation. Central nervous system toxicity includes respiratory depression, anxiety, insomnia, headache, apathy, drowsiness, dizziness, loss of concentration, confusion, tremors, convulsions, and coma. These effects usually appear within a few minutes to 24 hours after exposure, depending on the extent of exposure. In nonfatal exposures, the effects are usually transient, with rapid and complete recovery following cessation of exposure. Recovery from diazinon poisoning results from increased availability of active acetylcholinesterase either from synthesis of new enzyme, the spontaneous hydrolysis of the enzyme-phosphate ester complex, or treatment with atropine, a competitive antagonist of acetylcholine at muscarinic and central nervous system receptors, and with pralidoxime (2-PAM), a drug that regenerates inhibited acetylcholinesterase enzyme by displacing the diethylphosphoester bond that diazoxon forms at the active site (Aaron and Howland 1998; ATSDR 1996).

In some cases, however, diazinon may cause a condition known as the intermediate syndrome (Aaron and Howland 1998; WHO 1998). This syndrome occurs during apparent recovery about 24–96 hours after severe cholinergic crisis, and includes paralysis of the respiratory muscles, proximal limb, muscles, neck flexors, and motor cranial nerves. Diazinon has been tested for organophosphate-induced delayed neurotoxicity in chickens; results were negative (ATSDR 1996). No cases of delayed neuropathy from diazinon exposure have been reported (ATSDR 1996; WHO 1998).

Acetylcholinesterase activity is also present in erythrocytes where it is known as erythrocyte acetyl-cholinesterase. Both forms of acetylcholinesterase are produced by the same gene and are kinetically identical. In *in vitro* assays, erythrocyte and neural acetylcholinesterase are inhibited to roughly the same extent by exposure to diazinon and many other organophosphorous compounds with insecticidal activity;

The mechanism of action with regard to pancreatic toxicity in dogs and guinea pigs appears to be inhibition of butyrylcholinesterase in the pancreas and its smooth muscle sphincters, leading to ductal hypertension and cholinergic hyperstimulation of the acinar cells (Dressel et al. 1980; Frick et al. 1987).

C.4 Health Guidelines

ATSDR (1996) derived an intermediate inhalation MRL of 0.009 mg/m^3 for brain acetylcholinesterase inhibition diazinon based on a NOAEL of 0.46 mg/m^3 in a 21-day study in rats. An uncertainty factor of 30 was applied. The LOAEL (20% decrease in brain acetylcholinesterase) was 1.57 mg/m^3.

ATSDR (1996) derived an intermediate oral MRL of $2x10^{-4}$ mg/kg/day based on a NOAEL of 0.021 mg/kg/day for brain acetylcholinesterase inhibition in dogs given diazinon in their food daily for 13 weeks. An uncertainty factor of 100 was used. The LOAEL (31% decrease in erythrocyte and brain acetylcholinesterase) was 5.9 mg/kg/day.

EPA (IRIS 2003) does not have an online file for diazinon.

The EPA (2000) Office of Pesticide Programs derived acute and chronic RfDs of $2.5x10^{-3}$ and $2x10^{-4}$ mg/kg/day based on NOAELs for cholinesterase inhibition of 2.5 mg/kg/day (in rats) and 0.02 mg/kg/day in seven feeding studies (in rats and dogs), respectively. No additional FQPA safety factor was needed; the PADs are therefore the same as the RfDs.

NTP (2003) and IARC (2003) do not include diazinon in their listings. The EPA (2000) Office of Pesticide Programs classified diazinon as a *not likely human carcinogen* based on the lack of evidence of carcinogenicity in mice and rats.

C.5 Derivation of Target-Organ Toxicity Dose (TTD) Values

TTDs were not derived. The intermediate oral MRL based on neurological effects is appropriate for use as a chronic guidance value as well, and is the same as the chronic oral RfD developed by EPA (2000).