Disposition of Peer Review Comments for Interaction Profile for Selected Metallic Ions Identified in Waste Water from Unconventional Oil and Gas Extraction Activities: Sodium, Strontium, Barium, Iron, Manganese, Calcium, and Magnesium

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Agency for Toxic Substances and Disease Registry

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Peer reviewers for the third pre-public comment draft of the Interaction Profile for selected Metallic Ions Identified in Waste Water from Unconventional Oil and Gas Extraction Activities: Sodium, Strontium, Barium, Iron, Manganese, Calcium, and Magnesium were:

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Comments provided by Reviewer #1

General Comment

COMMENT 1: Overall, the authors did a very good job in putting together this Interaction Profile. There are only a few minor changes that I would recommend, as described below.

RESPONSE: *ATSDR thanks the Reviewer for the comment.*

ATSDR Charge Questions and Responses

Chapter 1

QUESTIONS:

1) Do you agree with those effects known to occur in humans as reported in the text? If not, please explain why and provide a copy of additional references you would cite and indicate where (in the text) these references should be included.
2) Are the effects only observed in animals likely to be of concern to humans? Why or why not? If you do not agree, please explain.
3) Have exposure conditions been adequately described? If you disagree, please explain.

COMMENT 2: The authors begin the Introduction by describing the primary purpose of this Interaction Profile and the reasons why this Profile was initiated. They then describe the studies on UOG waste water and the metallic cations identified in these samples: sodium, strontium, barium, iron, manganese, calcium, and maganesium. The authors then establish their rationale for focusing on exposure to these cations through drinking water rather than other routes of exposure. They then describe the process by which they identified appropriate literature and other sources for this Profile. They also listed critical effects for the seven elements of interest. I do agree with effects known to occur in humans as reported in the text. They do not describe effects only observed in animals in the introduction. Exposure conditions were adequately described and the rationale for focusing on drinking water is sound.

RESPONSE: *No response needed.*

Specific Comments on Chapter 1

COMMENT 3: In Table 1, under "Iron", the authors have listed "Sulfuric acid, iron salt (1:?)". What does the "1:?" refer to?

RESPONSE: *The parenthetical phrase was deleted in Table J-1 (formerly Table 1).*

COMMENT 4: In Table 1, under "Manganese", the authors have listed "Manganese, ion (Mn_4)". Should the 4 be superscript?

RESPONSE: *The parenthetical phrase was deleted in Table J-1 (formerly Table 1).*

Chapter 2

QUESTIONS:

1) Do the health effect conclusions made in Chapter 2 adequately reflect the findings in the published literature? If not, please suggest appropriate changes.
2) Were adequately designed human studies identified in the text, if available (i.e., good exposure data, sufficiently long period of exposure to account for observed health effects, adequate control for confounding factors)? Were the major study limitations sufficiently described in the text without going into lengthy discussions? If study limitations were not adequately addressed, please suggest appropriate changes.
3) Were adequately designed animal studies identified in the text (i.e., adequate number of animals, good animal care, accounting for competing causes of death, sufficient number of dose groups, and sufficient magnitude of dose levels)? If not, does the inadequate design negate the utility of the study? Please explain.
4) Were the animal species appropriate for the most significant toxicological endpoint of the study? If not, which animal species would be more appropriate and why?
5) Has adequate attention been paid to dose-response relationships for both human and animal data? Please explain.
6) Are you aware of any studies that are not included in the profile that may be important in evaluating the toxicity of the substance? Please provide a copy of each study and indicate where in the text each study should be included.
7) Are you aware of any studies that are not included in the profile that may be relevant to deriving MRLs for any of the substance isomers? Please provide a copy if this is a new reference.
8) Are the conclusions appropriate given the overall database? If not, please discuss your own conclusions based on the data provided and other data provided to you but not presented in the text.
9) Does the binary approach seem reasonable?

COMMENT 5: The health effect conclusions in Chapter 2 do adequately reflect published findings. Adequately designed human and animal studies were identified and described in sufficient but not excessive detail, including the limitations of those studies. The animal studies described did involve species appropriate for the toxicological endpoint for each metal. Adequate attention was paid to dose-response relationships where there was sufficient support from the published literature. I am not aware of any excluded studies that should have been included. Overall, the conclusions are appropriate given the body of literature that exists for all seven metals studied. The binary approach was reasonable for several reasons. Considering each metal in isolation would have been too superficial, while considering interactions between more than two metals at a time would have been too complicated.

RESPONSE: *No response needed.*

Chapter 3

QUESTIONS:

1) Is there adequate discussion of the complex interactive effects and other substances? Does the discussion concentrate on those effects that might occur at hazardous waste sites? Please explain and provide any additional references.
2) If interactive effects with other substances are known, does the text discuss the mechanisms of these interactions? Please explain and provide any additional references.
3) Is this approach reasonable?

COMMENT 6: There is adequate discussion in this chapter of the potential for interactive effects between substances identified in UOG waste water. The discussion does focus on effects that might occur involving hazardous waste, given that the seven metals considered were those identified in UOG waste water. With regards to known interactive effects with other substances, this issue is not pertinent to this profile, given that it specifically focused on metals identified in UOG waste water. This approach was reasonable in my opinion.

RESPONSE: *No response necessary.*

Chapter 4

QUESTIONS:
1) Are these conclusions reasonable? Please explain and provide any additional comments.
2) Is information provided based upon current understanding of the literature?

COMMENT 7: Conclusions summarized in this chapter are reasonable. They are based upon published studies using cultured celles, laboratory animals, and humans. When evidence is lacking to suggest that adverse effects of exposure to one metal is influenced by exposure to other metal, the authors state this explicitly. The information provided in this chapter is provided upon current literature.

RESPONSE: *No response necessary.*

Chapter 5

QUESTIONS:
1) Are you aware of any additional references that should be included? Please provide citations.
2) Please note the suggested location in text that new articles should be cited.

COMMENT 8: Overall, the references are appropriate, except for a few instances which I have noted under the Appendices—please see below.

RESPONSE: *The suggested articles were retrieved and examined and some changes in the appendices were made as noted below.*

Appendices A through I

Please provide comments on the content and presentation of each of the included appendices.
COMMENT 9: Overall the appendices are well constructed and presented. A few comments:

In the appendix on iron (D-1, line 14-15), the mention of "stimulator of iron transport (SFT) transporters" should be removed. The data on this transporter have not been replicated in the field and are widely viewed to be artifacts.

RESPONSE: *Mention of SFT transporters was removed as suggested by the Reviewer.*

COMMENT 10: In the appendix on manganese (E-1, line 20-21), it should be noted that DMT-1 may not mediate dietary manganese absorption. Mice with intestinal DMT-1 deficiency do not have defects in

Mn absorption or tissue levels. (Intestinal DMT1 is critical for iron absorption in the mouse but is not required for the absorption of copper or manganese. Shawki et al. Am J Physiol Gastrointest Liver Physiol. 2015 Oct 15;309(8):G635-47.)

RESPONSE: *The article was retrieved, examined, and the text was changed as per the Reviewer's comment.*

> However, more recent studies with mice with intestinal DMT-1 deficiency showed no defects in manganese absorption or tissue levels, indicating that other transport system can operate in the gastrointestinal tract (Shawki et al. 2015).

COMMENT 11: Also in the appendix on manganese (E-1, line 33-34), it should be noted that there is very strong evidence that ZIP8 and ZIP14 are essential for Mn transport in humans. The evidence for the other transporters listed is much weaker. The ZIP8 and ZIP14 evidence comes from reports of inherited diseases of Mn deficiency and excess respectively and studies of animal models of these diseases. (Genetic Disorders of Manganese Metabolism. Anagianni S, Tuschl K. Curr Neurol Neurosci Rep. 2019 May 14;19(6):33.)

RESPONSE: *The article was retrieved, examined, and the text was changed as per the Reviewer's comment.*

> Evidence that ZIP8 and ZIP14 are the most important transporters for cellular influx of manganese comes from studies of diseases of manganese deficiency and excess, respectively, and studies of animal models of these diseases (Anagianni and Tuschl 2019).

COMMENT 12: In A.3, line 21, what is meant by "saltwater homeostasis"?

RESPONSE: *"Saltwater homeostasis" was replaced by "sodium and water homeostasis" in the appendix with background information for sodium.*

> While disturbance in sodium and water homeostasis…

Annotated Comments

The Reviewer suggested a number of editorial revisions; most of the suggested revisions were made to the profile. Some stylistic changes that were purely arbitrary were not incorporated.

Comments provided by Peer Reviewer #2

ATSDR Charge Questions and Responses

Chapter 1

QUESTION: Do you agree with those effects known to occur in humans as reported in the text? If not, please explain why and provide a copy of additional references you would cite and indicate where (in the text) these references should be included.

COMMENT 1: It is unclear how Tables 2 and 3 were generated. I have provided some additional examples of toxic effects reported for some of the cations that are not considered in table 3 (there are many others). Indeed Tables 2 and 3 are so incomplete they are inadequate.

RESPONSE: *Text in Chapter 1 was revised to more clearly indicate how these tables 3 (now Tables 1 and 2) were generated, specifically that they were generated principally from the sources used to prepare Appendices A–G. ATSDR believes that information in the appendices sufficiently provides public health assessors (the intended audience for interaction profiles) with an accurate broad overview of pertinent knowledge on the toxicity of the individual metallic cations.*

Health guidance values for repeated oral exposure were identified by searching ATSDR (http://www.atsdr.cdc.gov/toxprofiles/index.asp), U.S. Environmental Protection Agency (EPA) Integrated Risk Information System (IRIS) (http://www.epa.gov/iris/index.html), and the National Academy of Sciences (NAS) (https://www.nap.edu) for minimal risk levels (MRL), reference doses/concentrations (RfD/RfC), and tolerable upper intake limits (UL), respectively. None of the subject metallic cations have been assessed for carcinogenicity by the International Agency for Research on Cancer (IARC; https://monographs.iarc.fr/agents-classified-by-the-iarc/) or the National Toxicology Program (NTP; https://ntp.niehs.nih.gov/whatwestudy/assessments/cancer/roc/index.html), and no cancer slope factors are available (see Appendices A–G). The EPA IRIS classified barium, manganese, and strontium in cancer Group D, not classifiable as to human carcinogenicity, and has not assessed the carcinogenicity of calcium, iron, magnesium, or sodium. Critical endpoints (i.e., the most sensitive effects) forming the basis of ATSDR MRL health guidance values for noncancer toxic effects from repeated oral exposure are tabulated in Table 1. Appropriate ATSDR oral MRL values were not available for some of the metallic cations: calcium, iron, magnesium, manganese, and sodium. For the first four cases of essential metals, health guidance values for repeated oral exposure derived by the NAS (ULs) were used to identify critical effects. For manganese, EPA's oral RfD was used (see Appendices A–G for additional details). The following critical effects were identified for repeated oral exposure to each metal of concern: barium (kidney effects), iron (gastrointestinal effects), calcium (kidney stones), magnesium (diarrhea), manganese (neurobehavioral effects), sodium (hypertension), and strontium (skeletal effects).

Based on the reviews in the ATSDR or NAS documents deriving the health guidance values, some biological systems are adversely affected by repeated exposure to more than one of the subject metallic cations (see Appendices A–G). Table 2 (prepared from information in Appendices A–G) describes common targets from repeated oral exposure (not necessarily the critical effects) as the cardiovascular system for barium, sodium, and potentially iron, the nervous system for barium, iron, and manganese, the gastrointestinal system for barium, iron, and magnesium, and the kidney for barium, calcium, magnesium, and potentially iron. Additional information on these toxic effects, as well as toxicokinetic and mechanistic considerations, are

presented in Appendices A–G. In addition, Toxicity Target Doses (TTDs) for less sensitive effects (i.e., effects occurring at doses higher than those associated with the critical effect for the health guidance value) were derived, if available dose-response data in the review documents were sufficient for derivations. Individuals at risk for primary or secondary iron overload due to altered iron absorption/uptake/excretion because of genetic or disease factors or extremely high oral intakes may develop systemic toxicity involving many organs, particularly the liver and heart, but available dose-response data were insufficient to derive TTDs for these high-dose iron effects (see Appendix C). Information included in the appendices is intended to provide public health assessors with a broad, accurate overview of the toxicity of individual compounds, and is based primarily on existing ATSDR Toxicological Profiles with supplementation from other public health, occupational health, or regulatory agency guidance documents (e.g., NAS, European Food Safety Authority [EFSA], IRIS, American Conference of Governmental Industrial Hygienists [ACGIH]). In instances where guidance documents were limited to older NAS documents (sodium, iron) and/or specific information was not included in available public health guidance documents (e.g., mechanisms of toxicity), reviews and meta-analyses from the peer-reviewed literature were included as appropriate. Appendices are not intended to provide a comprehensive review of the primary literature. Primary literature reviewed for this document was limited to studies focusing on possible interactions among the subject metallic cations.

In addition, Table 2 was modified to more clearly indicate cases for which adequate data were available for deriving TTDs for less sensitive effects. See revised profile to view revised table; data availability is summarized in revised table footnotes.

[a]Critical effects for derivation of health guidance values (MRLs, RfDs, or ULs) are bolded and italicized. Additional information on other health effects associated with higher exposures to the subject metallic ions is presented in Appendices A-G. Available data were adequate to derive TTDs for cardiovascular and neurological effects from repeated oral exposure to barium (Appendix A). For iron, data were inadequate to derive a TTD for neurotoxicity. For magnesium, data were adequate to derive a TTD for kidney effects (Appendix D). For manganese, data were adequate to derive a TTD for reproductive effects, but the resultant value was equivalent to the RfD. For the other cations, data were inadequate to derive oral TTDs for less sensitive effects occurring above the critical effect for the MRL, RfD, or UL (see Appendices A–G).
[b]Individuals susceptible to primary or secondary iron overload due to genetic disorder/polymorphism or disease state can develop systemic iron toxicity (cardiovascular, neurological, kidney, liver, or reproductive); limited evidence exists for iron overload symptoms in some populations with unusually high dietary intakes in food or drinking water. Available dose-response data were inadequate to derive TTDs for high-dose effects from iron (Appendix C).
[c]ATSDR (2012) did not derive a chronic or intermediate-duration oral MRL for manganese. The RfD for manganese (0.14 mg/kg/day) is recommended for ATSDR public health assessments in the absence of a chronic oral MRL. The RfD is similar in value to the NAS UL for manganese (0.16 mg/kg/day) and is similarly based on a lack of adverse effects associated with average manganese intake levels in Western diets (Appendix E).

QUESTION: Are the effects only observed in animals likely to be of concern to humans? Why or why not? If you do not agree, please explain.

COMMENT 2: Yes effects seen in animals are likely to be of concern to humans.

RESPONSE: *No response needed.*

QUESTION: Have exposure conditions been adequately described? If you disagree, please explain.

COMMENT 3: It is not clear why cancer effects were not considered. It's also unclear which cations are present in water and then the process used by ATSDR to identify the ones for study.

RESPONSE: *Changes to Chapter 1 text were made to emphasize that the subject cations represent metallic cations reported at elevated concentrations in UOG waste fluids in several research reports (as described in Appendix I of the profile).*

> For the focus of this Interaction Profile, ATSDR selected the seven metallic cations that have been detected in UOG extraction waste water samples at the highest elevated concentrations: barium, calcium, iron, magnesium, manganese, sodium, and strontium (see Appendix I). Relative toxicity was not considered in the selection of the metallic cations. ATSDR believed that it was important to evaluate evidence for interactions among each of the metallic cations, even though reported concentrations for cations with the highest values (calcium and sodium) were at least an order of magnitude higher than those metallic cations with the lowest concentrations in UOG extraction waste water samples (iron and manganese) (see Appendix I).

As discussed in Appendices A–G, cancer toxicity values (i.e., EPA cancer slope factors) have not been determined for any of the subject cations by the EPA, the agency on which ATSDR depends for these values. There is limited evidence for associations between high sodium intake and/or high sodium levels in urine and risk of gastric cancer (noted in Appendix F); hepatic iron overload conditions in humans and increased risk for liver cancer (as noted in Appendix C) and calcium dietary supplements and prostate cancer (Appendix B), but increased cancer risk from exposure to these cations is not supported by sufficient evidence to raise regulatory or public health concerns. Because of this, ATSDR is confident that, until other more convincing evidence is available, public health assessments for these cations will be based on their noncancer effects. Thus, the conclusions and recommendations made in Chapters 3 and 4 are for assessments of noncancer health risks from mixtures of these seven selected metallic cations, and whether or not evidence on interactions is currently sufficient to qualitatively modify component-based hazard index approaches for noncancer effects. Text has been added to Chapter 1 to clarify this focus.

> Noncancer effects from these cations were the focus of this profile because none of them have cancer weight-of-evidence determinations or cancer slope factors, which would be used in public health assessments (see Appendices A–G).
>
> None of the subject metallic cations have been assessed for carcinogenicity by the International Agency for Research on Cancer (IARC; https://monographs.iarc.fr/agents-classified-by-the-iarc/) or the National Toxicology Program (NTP; https://ntp.niehs.nih.gov/whatwestudy/assessments/cancer/roc/index.html), and no cancer slope factors are available (see Appendices A–G). The EPA IRIS classified barium, manganese, and strontium in cancer Group D, *not classifiable as to human carcinogenicity*, and has not assessed the carcinogenicity of calcium, iron, magnesium, or sodium.

Nevertheless, ATSDR did not restrict its literature search activities for interaction data to only noncancer effects or endpoints. Chapter 1 was revised to emphasize this point.

> The search objective was to identify noncancer and cancer toxicity, toxicokinetic, and interaction data from studies of humans and laboratory animals, as well as mechanistic studies using tissue, cell, or *in vitro* systems.

As a result, the profile described and evaluated, in Section 2.1, a fairly recent research report that samples of UOG waste fluids were cytotoxic to cultured human cells and could transform these cells so that they induced tumors when injected into mice (Yao et al. 2015). Even though the research investigators proposed that at least a portion of the transformational activity of the waste fluid could be hypothetically attributed to the cytotoxicity of metallic cations present in the waste fluids at high concentrations relative to other components, available evidence is inadequate to determine that repeated oral exposure to mixtures of the selected metallic cations will increase cancer risk in humans.

COMMENT 4: See text for additional comments. A significant concern relates to the literature search methods and inclusion and exclusion criteria. I'm also concerned that no rationale is provided for presenting the health effects of most concern. They appear to be based on endpoints identified for the derivation of toxicity values (e.g., MRL, RfD, other) – but this does not consider interactions between two or more cations. The document does not consider this factor (although it appears to have been part of the search strategy). I'm also concerned that shared mechanisms of action were not considered independent of the target organs (e.g., effects on reactive species generation may be informative). The authors may want to consider the addition of a figure, often refered to as a 'mineral wheel' that illustrates the complexity of the metal-metal interactions. I agree that references related to plants may not be relevant for evaluating the complex mixture and human health effects; however, it may also be worth at least mentioning that the use of this water for plant irrigation might lead to altered mineral uptake and could contribute to increased dietary intake.

RESPONSE: *Additional details were added to the description of the literature search in Appendix J, including the inclusion and exclusion criteria.*

> Information to prepare this profile was obtained via searches of the literature. The search objective was to identify noncancer and cancer toxicity, toxicokinetic, and interaction data from studies of humans and laboratory animals, as well as mechanistic studies using tissue, cell, or *in vitro* systems. An initial search of PubMed, Toxline, and Toxcenter was conducted in May 2015 to identify references with records mentioning two or more of five metals of interest (barium, iron, manganese, sodium, and strontium) using Chemical Abstracts Service Registry Numbers (CASRNs), Unique Ingredient Identifiers (UNIIs), and synonyms. In October 2018, an update, date-limited search of the same databases was conducted for these same five metallic cations, along with a non-date-limited search of PubMed for references with records mentioning calcium or magnesium and at least one other of seven metals of interest (the previously mentioned five metals, plus calcium and magnesium), augmented by a gray literature search in selected institutional websites: FDA, EFSA, INCHEM.org, eChemPortal, WHO, government of Canada, government of Japan, and Australia National Industrial Chemicals Notification and Assessment Scheme (NICNAS).
>
> A senior toxicologist manually screened titles and abstracts of the retained records to select potential studies of interest; 268 records were selected for retrieval and full text evaluation. The toxicologist selected interaction studies with whole-body exposure scenarios with mammals but did not exclude interaction studies conducted with isolated cell components, cells, or tissues.

More significantly, with respect to the literature search, targeted supplemental PubMed searches were conducted in October 2020 for individual binary combinations of the metallic cations, which the previous search protocol had indicated were data poor (barium and calcium; barium and iron; barium and magnesium; barium and manganese; barium and sodium; barium and strontium; iron and sodium; iron and strontium; magnesium and sodium; magnesium and strontium; manganese and sodium; manganese and strontium; and sodium and strontium). These individual PubMed searches linked the elements' names to either "transport proteins," "interactions," or "gastrointestinal absorption." Description of this search was added to Chapter 1:

> In addition, targeted supplemental PubMed searches were conducted in October 2020 for individual binary combinations of the metallic cations, which the previous search protocol had indicated were data poor (barium and calcium; barium and iron; barium and magnesium; barium and manganese; barium and sodium; barium and strontium; iron and sodium; iron and strontium; magnesium and sodium; magnesium and strontium; manganese and sodium; manganese and strontium; and sodium and strontium). These individual PubMed searches linked the elements' names to either "transport proteins," "interactions," or "gastrointestinal absorption." This search

was successful in identifying many studies of interactions among the cations and various membrane transport systems in isolated membrane vesicles, cells, or tissues that the previous protocol had missed. From these searches, about 240 additional reports were selected for full text evaluation; a preference for the most recent reviews was used for the supplemental searches identifying more than 300 reports (e.g., barium and calcium; barium and sodium; iron and sodium; magnesium and sodium; and manganese and sodium).

These supplemental searches were successful in identifying many studies of interactions among the cations and various membrane transport systems in isolated membrane vesicles, cells, or tissues that the previous protocol had missed. ATSDR is appreciative of the carefulness of the Reviewer; information added to Chapter 2 as a result of the supplemental searches makes the document more comprehensive and better able to make the recommendations in Chapter 3 for public health assessments for mixtures containing these metallic cations.

Changes were also made to Chapter 1 text and tables to better clarify that: (1) the critical endpoints (i.e., the most sensitive effects) on which ATSDR MRL health guidance values for repeated oral exposure are based, are tabulated in Table 1; (2) appropriate ATSDR oral MRL values were not available for some of the metallic cations: sodium, iron, calcium, magnesium, and manganese; (3) for the first four cases of essential metals, health guidance values for repeated oral exposure derived by the NAS (ULs) were used to identify critical effects; and (4) for manganese, EPA's oral RfD was used, as described in Appendices A–G.

Critical endpoints (i.e., the most sensitive effects) forming the basis of ATSDR MRL health guidance values for noncancer toxic effects from repeated oral exposure are tabulated in Table 1. Appropriate ATSDR oral MRL values were not available for some of the metallic cations: calcium, iron, magnesium, manganese, and sodium. For the first four cases of essential metals, health guidance values for repeated oral exposure derived by the NAS (ULs) were used to identify critical effects. For manganese, EPA's oral RfD was used (see Appendices A–G for additional details). The following critical effects were identified for repeated oral exposure to each metal of concern: barium (kidney effects), iron (gastrointestinal effects), calcium (kidney stones), magnesium (diarrhea)manganese (neurobehavioral effects), sodium (hypertension), and strontium (skeletal effects).

See revised profile to view revised Table 2; revised Footnotes from Table 2 are provided below.

[a]Critical effects for derivation of health guidance values (MRLs, RfDs, or ULs) are bolded and italicized. Additional information on other health effects associated with higher exposures to the subject metallic ions is presented in Appendices A-G. Available data were adequate to derive TTDs for cardiovascular and neurological effects from repeated oral exposure to barium (Appendix A). For iron, data were inadequate to derive a TTD for neurotoxicity. For magnesium, data were adequate to derive a TTD for kidney effects (Appendix D). For manganese, data were adequate to derive a TTD for reproductive effects, but the resultant value was equivalent to the RfD. For the other cations, data were inadequate to derive oral TTDs for less sensitive effects occurring above the critical effect for the MRL, RfD, or UL (see Appendices A–G).

[b]Individuals susceptible to primary or secondary iron overload due to genetic disorder/polymorphism or disease state can develop systemic iron toxicity (cardiovascular, neurological, kidney, liver, or reproductive); limited evidence exists for iron overload symptoms in some populations with unusually high dietary intakes in food or drinking water. Available dose-response data were inadequate to derive TTDs for high-dose effects from iron (Appendix C).

[c]ATSDR (2012) did not derive a chronic or intermediate-duration oral MRL for manganese. The RfD for manganese (0.14 mg/kg/day) is recommended for ATSDR public health assessments in the absence of a chronic oral MRL. The RfD is similar in value to the NAS UL for manganese (0.16 mg/kg/day) and is similarly based on a lack of adverse effects associated with average manganese intake levels in Western diets (Appendix E).

cMRL = chronic-duration Minimal Risk Level (Agency for Toxic Substances and Disease Registry); cRfD = chronic-duration reference dose (U.S. Environmental Protection Agency); cUL = chronic tolerable upper intake limit (National Academy of Sciences)

Chapter 2

QUESTION: Do the health effect conclusions made in Chapter 2 adequately reflect the findings in the published literature? If not, please suggest appropriate changes.

COMMENT 5: I have suggested a number of references – however my general sense is that the search terms used, inclusion and exclusion criteria etc did not support the conclusions regarding metal-metal interactions. In many cases I was able to quickly search the lietarture and find interactions at the cellular level. In some write-ups the ATSDR discussed mechanistic data therefore it's not clear why some mechanisms were considered while others were either not found or dismissed. As noted earlier, I am also concerned that the main targets identified for individual metals may not be appropriate for a cumulative risk assessment (or hazard assessment for a complex mixture). Its possible that secondary targets that are shared by multiple cations may become more important with a complex mixture.

RESPONSE: *ATSDR appreciates the recommendation from the Reviewer to incorporate more information regarding interactions at the cellular level. Sections of Chapter 2 that were indicated to be data poor (see Response to Comment 4) were revised to summarize these types of data (found in supplemental literature searches) and to add a discussion of their relevance to a principal issue of the profile addressed in Chapter 3: how to conduct cumulative public health assessments of repeated oral exposure to complex mixtures containing these seven metallic cations. The resulting revisions are too extensive to include in this response to comments. However, an example is given in Section 2.2.19 (Manganese and Sodium):*

> Interactions between manganese and other divalent cations at binding sites on sodium membrane transport systems in isolated membranes, cells, or tissues have been the subject of research for many years. Conditions under which manganese interacts with sodium transport systems have been described with isolated cell and membrane preparations from tissues including blockage of sodium/calcium exchange in embryonic cardiac cells (Mead and Clusin 1984); blockage of sodium ionic current in single canine cardiac Purkinje cells (Hanck and Sheets 1992; Sheets and Hanck 1992); inhibition of sodium-calcium exchange in sarcolemmal vesicles (Trosper and Philipson 1983) and smooth muscle cells in guinea-pig ureter tissue strips (Aickin et al. 1987); inhibition of exchange currents in guinea-pig heart ventricular myocytes (Kimura et al. 1987); inhibition of calcium uptake and sodium efflux in intact squid giant axons (Allen 1990); inhibition of the K^+-dependent Na^+-Ca^{+2} $^+$exchanger (NCKX2) and NCX1 in cultured Chinese hamster lung cells (CCL-39 cells; Uehara et al. 2005) and inhibition of Na^+ K^+-ATPase in human red blood cell membranes (Sachs 1988). In addition, the use of manganese-enhanced magnetic resonance imaging of the heart has been shown to be dependent on manganese accumulation and retention in the heart via the sodium-calcium exchanger in isolated perfused rat hearts (Chen et al. 2012). In addition, conditions under which divalent cations, including barium, calcium, and manganese, interact with isolated preparations of the Na^+/K^+-ATPase sodium pump have been described (Gatto et al. 2007; Robinson 1981).
>
> The physiological and toxicologic relevance of observations of manganese and sodium interactions at binding sites in transport proteins in isolated cell or tissues to environmentally relevant oral exposures to both cations, however, is unclear given the limited understanding of whole-body complex homeostatic systems for these metallic cations. Studies examining toxicokinetic endpoints (such as accumulation in toxicity target tissues), nutritional balance, or toxicological endpoints following repeated oral exposure of humans or laboratory animals to concomitant excess levels of manganese and sodium were not located and would be more useful for understanding how repeated oral exposure to excess levels of both manganese and sodium may influence each other's toxicity.

ATSDR agrees that it is possible that a component-based hazard index approach (discussed in Chapter 3) based on the critical targets for the individual metals may be less accurate than a hazard index approach due to joint toxic actions on secondary targets. That is why derivation of TTDs for each of the metals was attempted in preparing Appendices A–G. TTDs for all known targets (not just the critical target that forms the basis of the health guidance values for the individual metals) allows a hazard index approach based on a common target (e.g., the brain) or a common target through a shared mode of action (e.g., brain damage through reactive oxygen species). Unfortunately, available dose-response data in sources used to prepare the appendices were inadequate to derive TTDs for most of the metals. TTDs were developed for barium (Appendix C) in the draft presented to the Reviewer. In revising the draft document, additional data were located to develop TTDs for iron (see revised Appendix D) and magnesium (see revised Appendix G).

Discussion of the "data need" for additional data to derive TTDs was added to Section 3.3 and highlighted in each of the 21 subsections of Section 2.2.

The recommended component-based hazard index approach and binary evaluation of interaction data is acknowledged to be a practical approach with inherent uncertainty due to evidence that coupling of metallic cation homeostatic mechanisms is complex and can overlap for two or more metals. New studies comparing effects on pertinent endpoints for possible shared toxicity targets (e.g., neurological, cardiovascular, kidney) in laboratory animals orally exposed to mixtures of three or more of the subject metallic cations, with effects from exposure to the individual components alone may lead to better characterization of joint toxic actions on the selected endpoints and help to improve public health assessments for oral exposure to mixtures containing the metallic cations. Designs for these studies with such mixtures should consider the relative proportion of the metals in UOG extraction waste water and expectations on how those proportions may change after release into the environment.

Endpoints in such studies would be most useful if they were associated with putative shared toxicity targets among the subject metallic cations, including the nervous system, cardiovascular system, and the kidney.

Other new studies aimed at better describing dose-response relationships for less sensitive effects occurring at oral exposure levels to the individual metallic cations above the doses associated with the critical effects of health guidance values could decrease uncertainty in common-target hazard indices, especially for the various effects associated with high iron tissue accumulation (liver, kidney, nervous system, and cardiovascular endpoints) and for possible skeletal, neurological, and kidney effects from excess sodium salt.

Better assessment of neurological hazards is likely with results from appropriately designed studies examining brain tissue concentrations and neurological endpoints in laboratory animals orally exposed to mixtures of excess barium, iron, and manganese and comparing the responses to responses to those from sole exposure to the individual cations. Similarly designed studies may better inform joint toxic actions of barium, iron, and sodium in inducing hypertension; barium, calcium, iron, and magnesium in inducing kidney effects; and iron and magnesium in inducing gastrointestinal effects. Additional studies with laboratory animals may better inform whether or not supplemental calcium or magnesium may counteract sodium-salt induced hypertension, combined hypertension effects for barium, iron, and sodium, or combined neurotoxic effects from excess barium, iron, and manganese.

QUESTION: Were adequately designed human studies identified in the text, if available (i.e., good exposure data, sufficiently long period of exposure to account for observed health effects, adequate control for confounding factors)? Were the major study limitations sufficiently described in the text without going into lengthy discussions? If study limitations were not adequately addressed, please suggest appropriate changes.

COMMENT 6: In general, there are inadequate descriptions of key studies to make this evaluation.

RESPONSE: *It is beyond the scope of the interaction profile to provide a detailed description of individual studies. The interaction profile is intended to provide an overall discussion of the available data used to evaluate potential interactions in the mixture of concern.*

QUESTION: Were adequately designed animal studies identified in the text (i.e., adequate number of animals, good animal care, accounting for competing causes of death, sufficient number of dose groups, and sufficient magnitude of dose levels)? If not, does the inadequate design negate the utility of the study? Please explain.

COMMENT 7: Studies were inadequately described. I'm concerned that the ATSDR has not considered that animal studies may or may not be very informative since many rodent diets are oversupplemented. For example, rodent diets (e.g., older formulations of the NIH-07 diet) contained 100 ppm Mn – whereas the NRC indicates less than 10 ppm may be adequate. This is just one of several examples. Animal diets are often high in sodium, iron and other trace minerals (compared to levels found in human diet surveys for people on Western diets common in the US).

RESPONSE: *Regarding the comment about study descriptions, please see the Response to Comment 6. ATSDR agrees with the Reviewer that the nutrient content of the baseline diet is important to consider when evaluating the animal study results.*

QUESTION: Were the animal species appropriate for the most significant toxicological endpoint of the study? If not, which animal species would be more appropriate and why?

COMMENT 8: Animal studies were incompletely described. The inclusion/exclusion criteria were not mentioned. ATSDR cites both mammalian and avian studies. It does not appear that they considered zebrafish (or other) vertebrate studies – some of which have looked at mixtures.

RESPONSE: *See Response to Comment 6 with respect to description of animal studies. The revised text in Chapter 1 describes the selection criteria for literature screening:*

> The toxicologist selected with a preference for interaction studies with whole-body exposure scenarios with mammals but did not exclude interaction studies conducted with isolated cell components, cells, or tissues.

Information from studies of avian, aquatic, and mammalian species were used, including zebrafish, if found.

QUESTION: Has adequate attention been paid to dose-response relationships for both human and animal data? Please explain.

COMMENT 9: No the ATSDR does not uniformly consider dose-response data – indeed exposure doses are rarely provided in the documentation.

RESPONSE: *When available, ATSDR considered and described dose-dependence of interactions at the whole-body level of organization in Chapter 2. For many of the 21 pairs discussed in Chapter 2, however, studies designed to characterize possible interactions at this level of organization were not available. Examples in which dose-dependence of interactions at the whole-body level of interaction include:*

Section 2.2.9:

Competitive inhibitions between calcium and manganese for intestinal absorption mechanisms have been suspected for a long time, but inconsistent evidence for this possible interaction has been reported in studies of animals and humans. Observations in support of short-term mutual competition include decreased absorption of manganese in chickens fed high dietary intakes of calcium or calcium and phosphorus (Smith and Kabaija 1986; Wilgus and Patton 1939); decreased manganese retention from food in mice fed calcium-enriched food (or magnesium-enriched food) (Van Barneveld and Van den Hamer 1984); bone decalcification in rats and negative calcium balance in cows fed high dietary levels of manganese (Chornock et al. 1942; Reid 1947); and decreased absorption of ^{54}Mn in perfused rat intestinal sections in the presence of high concentrations of calcium (0.1 or 0.01 mM manganese in the presence of 0 or 1 mM calcium) (Lutz et al. 1993). The latter observation of calcium inhibition of manganese absorption was observed in perfused sections of proximal jejunum and colon, but a calcium stimulation of manganese absorption was observed in sections of the distal jejunum (Lutz et al. 1993). In 12-hour fasted human subjects, peak plasma manganese concentrations were markedly decreased when an oral dose of 40 mg manganese was accompanied by 800 mg of calcium as either calcium carbonate or 545 mL of 2% milk (Freeland-Graves and Lin 1991). In longer-term human nutritional balance studies, however, small, negative manganese balances after dietary calcium supplementation have been reported in some studies (McDermott and Kies 1987), but not in others (Price and Bunce 1972; Spencer et al. 1979).

Section 2.2.13:

Exposing rats to an iron-deficient and high-manganese diet (3.5 mg Fe/g and 100 mg Mn/kg) was reported to increase brain levels of oxygen stress indicators and alter performance results in a water maze behavioral test, compared with rats fed a control diet with 35 mg Fe/kg and 10 mg Mn/kg (Fitsanakis et al. 2009). Paradoxically, iron dietary supplementation also has been reported to increase manganese accumulation in the brains of rats (Chua and Morgan 1996; Fitsanakis et al. 2011), suggesting that the iron:manganese dietary ratio is important. Fitsanakis et al. (2011) concluded that both iron deficiency and iron supplementation can lead to increased manganese accumulation in the adult brain, and that iron supplementation may not necessarily counter manganese brain accumulation. Fitsanakis et al. (2011) presented a hypothetical explanation based on observations that high dietary iron levels can lead to degradation of ferroportin, a transport protein that is hypothesized to decrease the plasma iron levels allowing for increased manganese binding to transferrin and subsequent increased manganese transport to, and accumulation in, the brain. Tests of this mechanistic hypothesis have not been conducted. Nevertheless, a case-control study of iron-deficient (n=31) and control (n=36) infants found that iron-deficient infants had higher mean blood manganese concentrations than controls [2.555 versus 1.499 µg/L) and that iron therapy of 19 iron-deficient infants significantly decreased mean blood manganese concentrations, compared with pre-therapy values (2.971 versus 2.045 µg/L) (Park et al. 2013).

Revisions to sections identified as data poor in the earlier draft were revised, in response to comments from this Reviewer, to include studies of interactions at the cellular level; descriptions of dose-dependence of interactions at this level, however, were not provided, because the physiological and toxicological relevance of observation at this level in isolated cells or tissues to environmentally relevant oral exposure is unclear given the limited understanding of whole-body complex homeostatic systems for these metallic cations. Some examples of this dichotomy of describing dose-dependence of interaction in revised sections follows:

Section 2.2.6:

Barium, calcium, and strontium belong to Group (IIA) of the periodic table of elements. These elements have the same number of valence electrons and form stable divalent cations. The Stokes radius of Ba^{2+} is approximately 6% smaller than Ca^{2+} and Sr^{2+} (Kadhim and Gamaj 2020). These characteristics influence their interactions with binding sites on proteins, and extensive research has focused on their interactions with isolated membrane transport systems including: (1) components of mammalian calcium homeostatic systems including several types of calcium channels (see Sections 2.2.1 and 2.2.11); (2) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (3) TRP channels (Bouron et al. 2015); (4) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et al. 2012); and (5) calcium-activated SK potassium expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). Findings from these studies have been important to learning how the systems work in isolation, but the whole-body physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with respect to making reliable predictions of how combined oral exposure of humans or laboratory animals to any pair of metallic cations that are the subject of this profile may influence each other's toxicity.

Studies examining potential interactions between barium and strontium in whole-body mammals are few. Daily supplemental gavage administration of 33 mg Ba/kg/day plus 21.3 mg Sr/kg/day to young or old rats did not change concentrations of barium, calcium, or strontium in the tibia, compared with tibia concentrations in rats provided supplemental doses of barium or strontium alone (Panahifar et al. 2018). In an early study, gastrointestinal uptake of radiolabeled barium, calcium, radium, and strontium was measured in rats after single gavage administrations of the separate cations, but co-exposure was not part of the study (Taylor et al. 1962).

Section 2.2.11:

Calcium and strontium belong to Group (IIA) of the periodic table of elements. These elements have the same number of valence electrons and form stable divalent cations that have very similar Stokes radii (Kadhim and Gamaj 2020). These characteristics influence their interactions with binding sites on proteins and potential interactions between them have been investigated in a number of biological systems.

Many studies have examined the ability of strontium (and other divalent cations) to permeate through and modulate activity of isolated calcium transport systems (e.g., Bouron et al. 2015; Nelson 1986; Potreau et al. 1987; Tsien et al. 1987). In many systems, strontium is permeable (and could potentially compete with calcium) through calcium channels and can compete with calcium at allosteric binding sites. However, due to the complexity of calcium homeostasis at cellular, tissue, and whole-organism levels of organization, the significance of these types of findings to the possible joint action of calcium and strontium on toxicity targets after combined oral exposure of animals or humans is unclear.

Diets relatively high in strontium and low in calcium have been shown to disrupt calcium homeostasis in animals as evidenced by depressed intestinal absorption of calcium, depressed plasma calcium concentrations, and development of bone lesions (Bartley and Reber 1961; Corradino et al. 1971a, 1971b). In chickens, strontium inhibition of intestinal absorption of calcium involved strontium inhibition of the synthesis of calcitriol (1,25-dihydroxycholecalciferol) in the kidney, and strontium inhibition of calcium intestinal absorption was not evident in chickens fed a diet containing normal levels of calcium (Omdahl and DeLuca 1972). In basolateral membrane vesicles isolated from rat renal cortex, calcium was shown to be preferentially absorbed over strontium, demonstrating the discrimination of reabsorption processes in the renal proximal tubules between calcium and strontium; in the presence of 0.1 μM calcium, strontium significantly inhibited calcium uptake rates only when the molar ratio (Sr:Ca) was ≥16; in the presence of a high calcium concentration (1 μM), strontium concentrations up to 20 μM did not inhibit calcium uptake rates (Sugihira et al. 1992). The available evidence suggests that strontium can inhibit membrane transport of calcium only when external strontium concentrations are much higher than calcium concentrations.

Section 2.2.12:

Coupling between iron and magnesium homeostatic mechanisms has been demonstrated in studies of laboratory animals and isolated tissues and cells.

In short-term studies with isolated tissues and cells, iron uptake has been shown to be decreased by the presence of relatively higher concentrations of magnesium ions in incubating solutions. In isolated mouse intestinal mucosa fragments, the presence of 2, 5, or 10 mM magnesium ion in the incubating solution (containing 90 μM iron (III)) decreased iron uptake rates by about 25–30%, compared with medium containing no magnesium (Raja et al. 1987). The presence of 0.5 or 1 mM calcium had no significant effect on iron uptake rates (Raja et al. 1987). Iron transport from incubating solutions containing 20 μM iron (II) into rabbit erythroid cells was shown to be inhibited by the presence of exterior magnesium, with an IC_{50}=90μM, and stimulated by increased cytoplasmic levels of magnesium (Stonell et al. 1996). Stonell et al. (1996) proposed that iron uptake into erythroid cells may be mediated by a Na^{+}/Mg^{+2} antiport process.

The significance of this short-term inhibition of cellular uptake of iron by higher concentrations of magnesium to the long-term effects of repeated concomitant oral exposure is unclear, given the emerging understanding of the complex adaptability of homeostatic mechanisms for these essential metallic cations. It is conceivable that with long-term repeated oral co-exposure at levels below the respective tolerable upper intake levels, coordinated homeostatic mechanisms may adapt to maintain appropriate concentrations of both cations at the cellular and whole-body levels of organizations. Studies examining toxicokinetic endpoints (such as accumulation in toxicity target tissues), nutritional balance, or toxicological endpoints following repeated oral exposure of humans or laboratory animals to concomitant excess levels of iron and magnesium were not located and would be more useful for understanding how repeated oral exposure to excess levels of both cations may influence each other's toxicity.

Section 2.2.16:

Studies with isolated TRPM6 and TRPM7 channels, which are thought to be important components of magnesium cellular and organismal homeostasis, have shown that manganese can permeate through these channels, but the relevance of this possible competitive inhibition is of unknown physiological or toxicological significance (Bouron et al. 2015; Kolisek et al. 2019). Other studies indicate that magnesium and manganese can interact in complex ways with components of calcium homeostasis in isolated cells (see Sections 2.2.8 and 2.2.9), but the physiological and toxicological relevance of findings from this type of research is mostly unclear,

especially with regard to making reliable predictions of how combined oral exposure to excess magnesium and excess manganese may influence each other's toxicity.

Possible interactions between magnesium and manganese have been examined in a few oral exposure animal studies, but they do not present a clear understanding of interactions between these metallic cations at the whole-body level of organization. In mice, the short-term intestinal absorption of ^{54}Mn from gavage-delivered solutions containing 1 mM $MnCl_2$ was inhibited by concomitant exposure to relatively high concentrations of $MgCl_2$ (25 mM), but 2-week exposures to a magnesium-depleted diet did not influence short-term gastric absorption of ^{54}Mn (Van Barneveld and Van den Hamer 1984). In contrast, a study of rats fed a magnesium-deficient diet for 70 days reported that manganese absorption, measured as the difference between manganese oral intake and fecal excretion, was increased, compared with rats fed a magnesium-sufficient diet (Sanchez-Morito et al. 1999). It is uncertain if the apparent discrepancy between the two studies with respect to the effect of magnesium deficiency on the apparent absorption of manganese is due to differences in species, nutritional status, methods for measuring absorption, or some other factor, such as differences in metal concentrations in the diets used in the various studies. In other studies, sudden deaths occurred in pigs fed for up to 5 weeks a magnesium-deficient diet with a high level of manganese, whereas such deaths did not occur in pigs fed a sufficient magnesium diet with the same high manganese level (Miller et al. 2000, 2004). The deaths were associated with myocardial necrosis and mitochondrial swelling not seen in pigs fed sufficient magnesium and high manganese (Miller et al. 2004). No mechanistic studies were located that tested hypotheses related to molecular, cellular or tissue-level changes in manganese homeostatic components influenced by magnesium deficiency.

Section 2.2.21:

Interactions have been described in which sodium and strontium affect functions of isolated transport proteins, but the relevance of these interactions to how concomitant oral exposure to excess sodium and strontium may influence each other's toxicity is unknown. A few examples of reports of these types of interactions include competitive binding between calcium, strontium, and sodium at specific sites in an isolated sodium-calcium exchanger (Liao et al. 2016); inhibition of Na/K pump activity in isolated rat peritoneal mast cells by extracellular divalent cations including calcium, strontium, magnesium, and barium (Knudsen 1995); and modulation of gating activity of isolated sodium channels in lipid bilayers by divalent cations including calcium, barium, strontium, and magnesium (Cukierman and Krueger 1990).

Evidence for complex interactions between sodium and strontium have been demonstrated in a study that examined retention of sodium, phosphorus, and strontium in chickens provided varying levels of strontium and vitamin D3 (D3, Browning and Cowieson 2015). The results showed that supplemental strontium (500 or 1,000 mg Sr/kg diet) increased phosphorus, sodium, and strontium retention in birds fed 2,500 IU D3/kg diet kg, but reduced phosphorus, sodium, and strontium retention in birds fed 5,000 IU D3/kg diet. Although these results are consistent with strontium influencing the whole-body retention of sodium, their relevance to scenarios of co-exposure to excess sodium and strontium is unclear.

QUESTION: Are you aware of any studies that are not included in the profile that may be important in evaluating the toxicity of the substance? Please provide a copy of each study and indicate where in the text each study should be included.

COMMENT 10: I have provided numerous references – I did not provide copies of the studies since the way the search was performed does not appear to be uniform. I can quickly find multiple studies using

simple searches (e.g., strontium AND barium with another search term or two (e.g., MESH terms like toxicity; interactions, etc) and find literature that may be relevant – that seems to not have been considered). This is especially galring for mechanistic data where this data stream seems to be inconsistenctly considered by ATSDR. I have provided several examples in the text. These references may help identify hazards.

RESPONSE: *ATSDR thanks the Reviewer for the suggested references, which were retrieved and examined for inclusion in the revised text, and for the idea of conducting supplemental literature searches (see Responses to Comments 4, 5, and 13).*

QUESTION: Are you aware of any studies that are not included in the profile that may be relevant to deriving MRLs for any of the substance isomers? Please provide a copy if this is a new reference.

COMMENT 11: No I am not aware of studies that would contribute to deriving an MRL.

RESPONSE: *No response needed.*

QUESTION: Are the conclusions appropriate given the overall database? If not, please discuss your own conclusions based on the data provided and other data provided to you but not presented in the text.

COMMENT 12: I agree that there is rarely data to allow ATSDR to evaluate whether binary mixtures may have additive, synergistic, or other interactions. As I have mentioned earlier, I am concerned that only a select set of apical outcomes has been considered – when in reality many of the cations have multiple effects and may affect common cellular/enzymatic targets.

RESPONSE: *See Responses to Comments 4 and 5. In revising the document, ATSDR incorporated information from supplemental literature about interaction data at the cellular level for the pairs of metals identified as data poor in the previous draft and highlighted the need for adequate dose-response data for TTD derivations for less sensitive toxicity targets.*

QUESTION: Does the binary approach seem reasonable?

COMMENT 13: Yes, this is a reasonable way to evaluate the compounds. However, the ATSDR document does not adequately consider physicochemical (e.g., ionic radius) and other properties since cation interactions at the cellular level may reflect these physicochemical properties. This may help inform which binary pairs need additional attention. It's also not clear how cation interactions with known transporters (and other targets) were considered in the search. For example, DMT1 can help transport a number of cations.

RESPONSE: *See Responses to Comments 4 and 5, regarding supplemental searches for additional information about interactions at the cellular level.*

ATSDR agrees that physicochemical properties can be important to interactions at the subcellular and cellular levels of homeostatic mechanisms for these metallic cations and has added some notes in sections of Chapter 2 about this impetus to studying possible interactions at this level (as examples of these revisions see revised Sections 2.2.6 and 2.2.19 below). However, as noted in most, if not all, of the revised subsections of Chapter 2, the whole-body physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with regard to making reliable

predictions of how repeated combined oral exposure to any pair of metallic cations may influence their individual toxicity or indicating how they may jointly act on a shared toxicity target. For binary weights of evidence (BINWOEs) making a directional prediction, a small fraction (5%) was explained by ex vivo interactions (Pryzbyla et al. 2021). Examples of revised text in Chapter 2 follow.

Section 2.2.6, Barium and Strontium:

Barium, calcium, and strontium belong to Group (IIA) of the periodic table of elements. These elements have the same number of valence electrons and form stable divalent cations. The Stokes radius of Ba^{2+} is approximately 6% smaller than Ca^{2+} and Sr^{2+} (Kadhim and Gamaj 2020). These characteristics influence their interactions with binding sites on proteins, and extensive research has focused on their interactions with isolated membrane transport systems including: (1) components of mammalian calcium homeostatic systems including several types of calcium channels (see Sections 2.2.1 and 2.2.11); (2) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (3) TRP channels (Bouron et al. 2015); (4) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et al. 2012); and (5) calcium-activated SK potassium expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). Findings from these studies have been important to learning how the systems work in isolation, but the whole-body physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with respect to making reliable predictions of how combined oral exposure of humans or laboratory animals to any pair of metallic cations that are the subject of this profile may influence each other's toxicity.

Studies examining potential interactions between barium and strontium in whole-body mammals are few. Daily supplemental gavage administration of 33 mg Ba/kg/day plus 21.3 mg Sr/kg/day to young or old rats did not change concentrations of barium, calcium, or strontium in the tibia, compared with tibia concentrations in rats provided supplemental doses of barium or strontium alone (Panahifar et al. 2018). In an early study, gastrointestinal uptake of radiolabeled barium, calcium, radium, and strontium was measured in rats after single gavage administrations of the separate cations, but co-exposure was not part of the study (Taylor et al. 1962).

Section 2.2.19, Manganese and Sodium:

Interactions between manganese and other divalent cations at binding sites on sodium membrane transport systems in isolated membranes, cells, or tissues have been the subject of research for many years. Conditions under which manganese interacts with sodium transport systems have been described with isolated cell and membrane preparations from tissues including blockage of sodium/calcium exchange in embryonic cardiac cells (Mead and Clusin 1984); blockage of sodium ionic current in single canine cardiac Purkinje cells (Hanck and Sheets 1992; Sheets and Hanck 1992); inhibition of sodium-calcium exchange in sarcolemmal vesicles (Trosper and Philipson 1983) and smooth muscle cells in guinea-pig ureter tissue strips (Aickin et al. 1987); inhibition of exchange currents in guinea-pig heart ventricular myocytes (Kimura et al. 1987); inhibition of calcium uptake and sodium efflux in intact squid giant axons (Allen 1990); inhibition of the K^+-dependent Na^+-Ca^{+2} $^+$exchanger (NCKX2) and NCX1 in cultured Chinese hamster lung cells (CCL-39 cells; Uehara et al. 2005) and inhibition of Na^+,K^+-ATPase in human red blood cell membranes (Sachs 1988). In addition, the use of manganese-enhanced magnetic resonance imaging of the heart has been shown to be dependent on manganese accumulation and retention in the heart via the sodium-calcium exchanger in isolated perfused rat hearts (Chen et al. 2012). In addition, conditions under which divalent cations, including barium, calcium, and manganese, interact with isolated preparations of the Na+/K+-ATPase sodium pump have been described (Gatto et al. 2007; Robinson 1981).

The physiological and toxicologic relevance of observations of manganese and sodium interactions at binding sites in transport proteins in isolated cell or tissues to environmentally relevant oral exposures to both cations, however, is unclear given the limited understanding of whole-body complex homeostatic systems for these metallic cations. Studies examining toxicokinetic endpoints (such as accumulation in toxicity target tissues), nutritional balance, or toxicological endpoints following repeated oral exposure of humans or laboratory animals to concomitant excess levels of manganese and sodium were not located and would be more useful for understanding how repeated oral exposure to excess levels of both manganese and sodium may influence each other's toxicity.

In deriving health guidance values to protect against the development of adverse effects from repeated oral exposure, the critical effects for exposure to manganese are neurological, whereas the critical effect of exposure to excess sodium is increased blood pressure (see Appendices E and F). Overlapping toxicity targets across a range of exposure conditions have not been clearly identified for manganese and sodium, and TTDs for less sensitive effects from these metallic cations were not derived due to inadequate data (Appendices E and F.

Section 4 (Conclusions) was also revised to include a statement about the limitations of in vitro studies for predicting the direction of interactions of binary mixtures:

The physiological and toxicological relevance of the findings from *in vitro* studies is not always clear, especially with regard to making reliable predictions of how repeated combined oral exposure to any pair of metallic cations may influence their individual toxicity or indicate how they may jointly act on a shared toxicity target. For binary weights of evidence (BINWOEs) making a directional prediction, a small fraction (5%) was explained by *ex vivo* interactions (Pryzbyla et al. 2021).

Chapter 3

QUESTION: Is there adequate discussion of the complex interactive effects and other substances? Does the discussion concentrate on those effects that might occur at hazardous waste sites? Please explain and provide any additional references.

COMMENT 14: See document for some suggestions.

RESPONSE: *See Responses to "Annotated Comments" below.*

QUESTION: If interactive effects with other substances are known, does the text discuss the mechanisms of these interactions? Please explain and provide any additional references.

COMMENT 15: Formation of calcium oxalate uroliths is a complicated process that in veterinary species (e.g., dog) has some nutritional components (e.g., see Lulich et al 2001; Lekcharoensuk et al., 2001; others) – enough so that dietary levels of magnesium, phosphorus, and calcium levels have changed over time to reduce urolith formation. It's not clear whether this type of data was considered – and 'dismissed' because human evidence was available. As mentioned before methods and decisions used to evaluate possible data streams were poorly described at times.

RESPONSE: *The suggested articles were retrieved and examined. The complexity of factors involved in calcium oxalate uroliths/ kidney stone formation in humans and rats was discussed in Appendix B, Section B.3 and was based on authoritative reviews by NAS (2011) and EFSA (2012).*

The revised version was amended to add some of the information from the study by Lekcharoensuk et al. (2001) suggesting changes in several dietary components to reduce the risk of calcium oxalate uroliths in cats.

In Appendix B, Section B.2, discussion of the multiple factors was also amended with information from other recent reviews of stone disease in humans:

> ***Nephrolithiasis (Kidney Stones).*** NAS (2011) concluded that the best human evidence for calcium-induction of kidney stones comes from a large, well-designed cohort study of bone fracture frequency, bone density, and kidney stone frequency in 36,000 postmenopausal women (ages 50–79 years) who received a placebo or a supplement of 1,000 mg Ca/day and 400 IU vitamin D/day for up to 5 years (Jackson et al. 2006). The total average calcium intake for the treated group was about 2,100 mg Ca/day. The reported hazard ratio for kidney stones in the treated group was 1.17 (95% confidence interval [CI] 1.02–1.34), compared with the placebo group (449 cases in treated group versus 381 cases in the placebo group). NAS (2011) noted that earlier, smaller trials of calcium supplementation in older subjects reported no evidence for a link with nephrolithiasis (Borghi et al. 2002; Levine et al. 1994; Riggs et al. 1998; Williams et al. 2001). NAS (2011) also noted that a series of studies of younger women (Curhan et al. 1997, 2004) or men between 40 and 75 years old (Curhan et al. 1993) did not find consistent evidence for an association with kidney stone formation and provided limited evidence that taking calcium supplements with food may suppress the formation of kidney stones.
>
> In an independent review of the available data on possible associations between high calcium intakes and kidney effects, including kidney stones, EFSA (2012) concluded that: (1) calcium intakes up to about 2,400 mg Ca/day have not been associated with an increased risk of chronic hypercalciuria or impaired kidney function, and (2) calcium intakes up to 3,000 mg Ca/day have not been associated with an increased risk of nephrolithiasis in the general adult population. The EFSA (2012) Panel noted that the evidence for an association between calcium dietary supplementation and kidney stones presented by Jackson et al. (2006) was not found in a subsequent analysis of the same study; Wallace et al. (2011) found that when only subjects with stone outcomes during the time of treatment ("during active adherence") were included in the analysis, the increased risk estimate was of a similar magnitude (as in the earlier analysis, but was not statistically significant (hazard ratio 1.21; 95% CI 0.98–1.34). The EFSA (2012) Panel concluded that the Jackson et al. (2006) study "did not provide evidence for an increased risk of kidney stones which could be attributed to high calcium intakes."
>
> Other recent reviews present evidence that kidney stone disease in humans has multiple risk factors including sex, age, race, family history, several dietary factors other than calcium (e.g., higher dietary intakes of animal protein, sugar, or oxalates may increase risk and higher potassium or phytates may decrease risk), systemic disorders (e.g., obesity and diabetes increase risk), and fluid intake (e.g., low fluid intake increases risk) (Curhan 2007; Prochaska et al. 2016). The multiplicity of risk factors reflects the inconsistency among epidemiology studies finding positive associations between calcium intake and kidney stones. A number of prospective epidemiology studies have now consistently found an inverse relationship between dietary calcium and kidney stone risk, but intake of calcium supplements has been found to present a 20% added risk for kidney stones in older women, but not in younger women (Prochaska et al. 2016).

Appendix B, Section B.3:

> The mechanisms by which high-level calcium intake may produce kidney effects, such as kidney stones (nephrolithiasis) or nephrocalcinosis, are poorly understood. Nephrocalcinosis has not

been clearly associated with high intakes of calcium dietary supplements in humans, presumably due to the efficacy of complex homeostatic mechanisms for calcium but was associated with high calcium intakes by preadolescent rats (NAS 2011; Peterson et al. 1996). Symptoms associated with nephrocalcinosis are similar to those associated with renal dysfunction, such as painful and frequent urination, nausea, vomiting, or swelling. Although nephrocalcinosis has been proposed to be linked to the formation of kidney stones (in humans, the principal component of kidney stones is calcium oxalate), Vervaet et al. (2009) have proposed that nephrocalcinosis and calcium nephrolithiasis are independent pathologies associated with multiple factors including compromised or surpassed crystal clearance mechanisms and crystal adherence differences between normal renal epithelial cells and dedifferentiated or regenerating renal epithelial cells. Reflecting this putative mechanistic complexity, some human studies have reported statistically significant associations between high calcium intakes and increased risk for kidney stones (e.g., Jackson et al. 2006) and others have not found significant positive associations (Borghi et al. 2002; Levine et al. 1994; Riggs et al. 1998; Williams et al. 2001). Other studies of humans (Curhan et al. 1993, 1997, 2004), rodents (Mourad et al. 2006), and cats and dogs (Lekcharoensuk et al. 2001a, 2001b; Lulich et al. 2016) have observed inverse relationships between calcium dietary intakes and kidney stone risk (less risk with higher dietary calcium) and provided evidence that other components of the diet can influence the apparent association between high calcium intakes and formation of kidney stones (e.g., increased intakes of oxalates and sugar increase risk and increased intakes of potassium and phytates may decrease risk). A postulated mechanistic explanation for the apparent discrepancy between dietary calcium and supplemental calcium is that dietary calcium may inhibit oxalate absorption in the gut more effectively than supplementary calcium not taken with meals (Prochaska et al. 2016). Overall, the mechanism of kidney stone formation in humans is not clearly understood, but multiple risk factors other than calcium intake from supplements are widely recognized, including low fluid intake, increased urinary calcium and oxalate excretion, increased dietary sugar, high body mass index, and type II diabetes (Curhan 2007; EFSA 2012; NAS 2011; Prochaska et al. 2016).

The complexity of deciding whether or not studies on dietary calcium supplementation and calcium urolith formation should form the basis of a public health guidance value for repeated oral exposure to supplemental or excess calcium is also discussed at length in Appendix B.

QUESTION 16: Is this approach reasonable? As a screening method it may be appropriate.

COMMENT: I am concerned that some 'cherry picking' of toxicity values was considered – e.g., why use a UL vs MRL etc. In addition, the derivation of TTD's is incompletely provided.

RESPONSE: *Text was revised in Chapter 1, Appendices, and Chapter 3 to better clarify that ULs or RfDs were only recommended for use in the hazard indices only when ATSDR MRLs were not available.*

Chapter 1:

Health guidance values for repeated oral exposure were identified by searching ATSDR (http://www.atsdr.cdc.gov/toxprofiles/index.asp), U.S. Environmental Protection Agency (EPA) Integrated Risk Information System (IRIS) (http://www.epa.gov/iris/index.html), and the National Academy of Sciences (NAS) (https://www.nap.edu) for minimal risk levels (MRL), reference doses/concentrations (RfD/RfC), and tolerable upper intake limits (UL), respectively. None of the subject metallic cations have been assessed for carcinogenicity by the International Agency for Research on Cancer (IARC; https://monographs.iarc.fr/agents-classified-by-the-iarc/) or the National Toxicology Program (NTP; https://ntp.niehs.nih.gov/whatwestudy/assessments/cancer/roc/index.html), and no cancer slope

factors are available (see Appendices A–G). The EPA IRIS classified barium, manganese, and strontium in cancer Group D, *not classifiable as to human carcinogenicity*, and has not assessed the carcinogenicity of calcium, iron, magnesium, or sodium. Critical endpoints (i.e., the most sensitive effects) forming the basis of ATSDR MRL health guidance values for noncancer toxic effects from repeated oral exposure are tabulated in Table 1. Appropriate ATSDR oral MRL values were not available for some of the metallic cations: calcium, iron, magnesium, manganese, and sodium. For the first four cases of essential metals, health guidance values for repeated oral exposure derived by the NAS (ULs) were used to identify critical effects. For manganese, EPA's oral RfD was used (see Appendices A–G for additional details).

Appendix B:

Because ATSDR MRLs and EPA RfDs for calcium have not been developed, the NAS (2011) ULs for calcium (based on increased risk for kidney stones in postmenopausal women taking dietary calcium supplements) are recommended to be the basis for provisional orals MRLs in ATSDR public health assessments of water-soluble forms of calcium. Thus, the NAS (2011) UL for adults 19–50 years old, 2,500 mg/day, is divided by a reference body weight of 70 kg to derive a surrogate oral MRL for soluble forms of calcium of 36 mg Ca/kg/day. For use in screening level assessments, it is thought that this oral MRL would be suitable for intermediate- and chronic-duration exposure scenarios. Other lifestage-specific oral MRLs could be derived similarly from age-group-specific NAS (2011) ULs, using appropriate reference body weights.

Appendix C:

The NAS (2001a) ULs for iron are recommended as intermediate- or chronic-duration surrogate MRLs for use in public health assessments, in the absence of ATSDR MRLs or an EPA RfD for oral exposure to iron compounds.

Appendix D:

Because ATSDR MRLs and EPA RfDs for magnesium have not been developed, the NAS (1997) UL for magnesium, 350 mg Mg/day (5 mg Mg/kg/day assuming a body weight of 70 kg), is recommended to be the basis for a provisional surrogate intermediate-duration oral MRL for adults in ATSDR public health assessments of magnesium compounds that are readily dissociated in the human body, including magnesium chloride, magnesium sulfate, magnesium acetate, magnesium hydroxide, and magnesium oxide.

Appendix E:

For oral exposure, the Institute of Medicine of the NAS recommended AI values for manganese of 2.3 and 1.8 mg/day for adult men and women, respectively (0.03 mg/kg/day assuming 70-kg body weight) (NAS 2001b). Based on a lack of adverse effects associated with manganese intake levels in Western diets, the Institute of Medicine of the NAS set a UL of 11 mg/day (0.16 mg/kg/day assuming 70-kg body weight) for manganese intake (NAS 2001b). Similarly, the EPA derived an RfD value of 0.14 mg/kg/day based on a lack of adverse effects at RDAs (IRIS 2002a) (see Table H-1 in Appendix H). Although the ATSDR (2012) did not derive a MRL for repeated oral exposure to manganese, the NAS UL (NAS 2001b) and EPA RfD (IRIS 2002a) guidance values are similar and represent reasonable estimates of acceptable oral intakes of manganese. For ATSDR public health assessments of chronic oral exposure to manganese, the EPA RfD is recommended.

Appendix F:

The Institute of Medicine of the NAS has determined that there is insufficient evidence of sodium toxicity risk within the apparently health population to establish a sodium tolerable upper limit (NAS 2019). The EPA has a drinking water advisory of 20 mg/L for individuals restricted to a

dietary intake of 500 mg sodium/day (EPA 2012). Due to lack of a clear relationship between sodium levels in drinking water and risk of hypertension, WHO did not establish a drinking water quality guideline level for sodium (WHO 2008).

ATSDR (2015) and EPA (IRIS 2019) have not derived noncancer toxicity values for sodium. The EPA (IRIS 2019), IARC (2015), and NTP (2014) have not assessed sodium for carcinogenicity.

Chapter 3:

In the absence of chronic oral MRLs for several of the essential metals, the NAS ULs are recommended for calcium, iron, magnesium, and sodium, and the RfD for manganese is recommended to be used in calculating the hazard index (see Appendices B, C, D, and F). The values of the NAS UL and the EPA RfD for manganese are similar in value and are similarly based on the absence of neurological effects in the general population with normal manganese dietary intakes (see Appendix E).

Text was added to each subsection of Chapter 2 (when missing) to note the ability to derive TTDs (or lack of adequate data, as is the case for most of the subject metals). For example:

Section 2.2.3:

In deriving health guidance values to protect against the development of adverse effects from repeated oral exposure, the critical effects for exposure to barium are kidney effects (nephropathy, Appendix A), whereas the critical effect for exposure to magnesium is mild diarrhea (a gastrointestinal effect, Appendix D). There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome via a common mode of action. But the kidney represents a potential common toxicity target when magnesium levels of exposure are sufficiently elevated (see Appendix D for description of a TTD for kidney effects from magnesium). TTDs were derived for cardiovascular and neurological effects from barium exposure levels higher than those associated with kidney effects but repeated oral exposure to magnesium has not been associated with these toxicity targets (see Appendices A and D).

Summary. Studies conducted in subcellular systems, isolated cells, and tissues have found evidence for various interactions between barium and magnesium on membrane transport of ions, neurotransmitters, and hormones. However, available interaction data are inadequate to determine whether or not repeated concomitant oral exposure to excess barium and magnesium may influence the adverse effects of barium on the cardiovascular and nervous systems or the effects of magnesium on the gastrointestinal tract, or whether their joint actions on the kidney may be additive, less-than-additive, or more-than-additive.

Chapter 4

QUESTION: Are these conclusions reasonable? Please explain and provide any additional comments.

COMMENT 17: I have raised multiple concerns regarding over reliance on a small set of toxic endpoints, not considering mechanistic data etc. The conclusions are reasonable for the descisions made by ATSDR to largely ignore these and other considerations; however, this draft document does not provide sufficient data to support the conclusions.

RESPONSE: *ATSDR thanks the Reviewer for articulating their concerns and thinks the revisions made in Chapter 1 (describing the literature searches; see responses to Comments 4 and 5) and Chapter 2 (using information from the supplemental literature searches and the additional specific references suggested by the Reviewer; see responses to Comment 9, for example) make the document better able to support the conclusions in Chapters 3 and 4.*

QUESTION: Is information provided based upon current understanding of the literature?

COMMENT 18: It's difficult to judge this since the search terms etc used in the literature search are not provided. The ATSDR indicates that: "Appendices are not intended to provide a comprehensive review of the primary literature, as primary literature reviewed for this document was limited to studies focusing on possible interactions among the subject metallic cations." How this goal was met remains unclear.

RESPONSE: *As described in the Responses to this Reviewer's Comments 4 and 5, revisions were made to better describe the literature searches in Chapter 1 and supplemental literature searches were conducted to identify more studies of interactions at the cellular level with incorporation for relevant information in subsections of Chapter 2 that were identified as data poor in the previous draft.*

Chapter 5

QUESTIONS: Are you aware of any additional references that should be included? Please provide citations.

COMMENT 19: See comments in main document.

RESPONSE: *See responses to these comments in Annotated Comments section below.*

QUESTION: Please note the suggested location in text that new articles should be cited.

COMMENT 20: See comments in main document.

RESPONSE: *See responses to comments in Annotated Comments section below.*

Appendices A through I

Please provide comments on the content and presentation of each of the included appendices.

COMMENT 21: Each appendix provides a brief summary of the toxicokinetics, toxicity, mechanism of action, and health guidelines for each individual cation. In general, the mechanism of action data is incomplete and does not provide an adequate overview of cellular/enzymatic etc targets – especially those that are affected by multiple cations of interest.

RESPONSE: *The "mechanism of action" sections of Appendices A–G are only meant to be a brief summary overview and were not changed in response to this comment. However, as noted in Responses to Comments 4 and 5 from this Reviewer, information from supplemental literature searches was added to Chapter 2 to highlight information about cellular-level interactions between pairs of the subject metals identified as data poor in the previous draft, and a discussion was added to Chapter 3 of potentially*

shared targets for several of the subject metals, even if data were inadequate to derive TTDs (e.g., discussion of potential nervous system toxicity from excess iron was added to Appendix C and added in Sections 2.2.1 and 3.2).

Annotated Comments

The Reviewer suggested a number of editorial revisions, most of the suggested revisions were made to the profile. Some stylistic changes that were purely arbitrary were not incorporated. Responses to Reviewer comments that were not considered editorial or stylistic are presented below.

COMMENT 22: Referring to the statement in the Introduction—..ATSDR considered the preparation of an Interaction Profile on repeated combined exposure to methane and metallic ions in drinking water.—the Reviewer commented "I found this confusing since methane exposure is not considered. It may be clearer to state this profile only addresses the metallic ions – but recognizes a broader concern exists."

RESPONSE: *ATSDR thinks that the historical development of the focus of the profile should be outlined in Chapter 1. To avoid confusion, however, the last sentence of the second paragraph of Chapter 1 was modified to foreshadow the conclusion reached in the historical development of the focus:*

> ATSDR initially considered the preparation of an Interaction Profile on repeated combined exposure to methane and metallic ions in drinking water but settled on a focus on interactions among metallic cations. Methane, although presenting a flammability and explosive hazard, is generally considered to be inert, and available scientific data are inadequate to establish a specific health hazard of concern for methane (other than as an asphyxiant gas) or evaluate whether co-exposure to metals may affect the expression of methane's toxicity or whether methane may affect the toxicity of metallic cations, as further explained in the following paragraphs.

COMMENT 23: Referring to the statement in the Introduction—… drinking water wells in Pennsylvania and Texas (overlying the Marcellus and Barnett Shales, respectively) in which gas concentrations were increasing...—the Reviewer made the comment "Which gases? Short hydrocarbons?"

RESPONSE: *The text in Chapter 1 was revised to clarify which gases were increasing:*

> Another study identified subsets of drinking water wells in Pennsylvania and Texas (overlying the Marcellus and Barnett Shales, respectively) in which methane, ethane, or propane concentrations were increasing with time (Darrah et al. 2014).

COMMENT 24: Referring to the statement in the Introduction—.. > magnesium (Mg, ~75–630) > iron (Fe, ~25–75) > manganese (Mn, ~4–45)—the Reviewer made the comment "Consider indicating that this does not consider toxicity only a rank order of concentrations."

RESPONSE: *The paragraph in Chapter 1 describing the final focus of the profile was revised:*

> For the focus of this Interaction Profile, ATSDR selected the seven metallic cations that have been detected in UOG extraction waste water samples at the highest elevated concentrations: barium, calcium, iron, magnesium, manganese, sodium, and strontium (see Appendix I). Relative toxicity was not considered in the selection of the metallic cations. ATSDR believed that it was important to evaluate evidence for interactions among each of the metallic cations, even though reported concentrations for cations with the highest values (calcium and sodium) were at least an

order of magnitude higher than those metallic cations with the lowest concentrations in UOG extraction waste water samples (iron and manganese) (see Appendix I).

COMMENT 25: Referring to the statement in the Introduction—Pennsylvania has only 6 deep injection wells, whereas Ohio has 177 and Texas has 50,000 (Brown 2014)—the Reviewer made the comment "Does the type of extraction technology limit the applicability of the profile to other sites? If so this could be made clearer in the title of the assessment."

RESPONSE: *ATSDR thinks the title is appropriate, as is, because selection of the metals of concern was based on data published for waste water from unconventional oil and gas extraction (Appendix I). ATSDR agrees, however, that the recommendations for assessing cumulative risks for mixtures with these metallic cations could be applied to mixtures obtained via some other contamination process.*

COMMENT 26: Referring to the statement in the Introduction—presence of methane in groundwater may influence potential toxic effects of metals or water chemistry (e.g., solubility of metals)—the Reviewer made the comment "Methane is classically considered relatively inert and its toxic effects are secondary to being an asphyxiant gas. Is this worth mentioning here?"

RESPONSE: *The following sentence was added to Chapter 1:*

ATSDR decided that including methane in an Interaction Profile for potential mixtures in waste water generated from gas extraction activities would not be useful, because the human health hazards associated with repeated exposure to methane gas by inhalation or methane gas dissolved in water are not well characterized. Associations between repeated exposure of humans or laboratory animals to methane and specific health hazards have not been established.

COMMENT 27: Referring to the statement in the Introduction—Yao et al. (2015) proposed that metallic cations present in the test substance filtrates could account for a considerable fraction of the observed *in vitro toxicity*—the Reviewer made the comment "They also indicated that "However, based on the data we gathered, we are not able to tell whether the malignant transformation activity was solely from Ba, Sr or other metals that have been detected in the flow back waters at lower levels." for example vanadium arsenic, or vanadium also cause these transformations."

RESPONSE: *Text was revised in Chapter 1 to better reflect concluding statements from Yao et al. (2015):*

Yao et al. (2015) proposed that metallic cations present in the test substance filtrates could account for a considerable fraction of the observed *in vitro* toxicity but noted that they could not discern if the "transformation activity was solely from Ba, Sr, or other metals that have been detected in the flow back waters at lower levels."

COMMENT 28: Referring to the statement in the Introduction—and several of these cations are essential elements that are only toxic at very elevated intakes (i.e., iron, calcium, and magnesium)—the Reviewer made the comment "How was carcinogenesis considered since ATSDR cites Yao et al 2015? Many of these cations are of unknown carcinogenic potential or would not be considered as carcinogenic. Did ATSDR consider other cations that may be of higher carcinogenic potential? How do these metals interact with known human carcinogens like arsenic?"

RESPONSE: *ATSDR did not consider other metallic cations that may be of higher carcinogenic potential, because they were not included in the list of cations detected in published accounts of the chemical compositions of samples of waste water from UOG extraction activities (Appendix I).*

COMMENT 29: Referring to the statement in the Introduction regarding the search criteria—..using Chemical Abstracts Service Registry Numbers (CASRNs) or synonyms—the Reviewer made the comment "This is an incomplete description of search terms and methods. Provide search terms used (e.g., what streams of evidence were considered: human, animal, in vitro; outcomes, MESH terms etc)."

RESPONSE: *Appendix J was added to the profile, which includes all search terms and methods:*

> Appendix J. Database Query Strings for Combinations of Selected Metallic Ions (Barium, Calcium, Iron, Magnesium, Manganese, Sodium, and Strontium)

COMMENT 30: The Reviewer made the following comment in the Introduction regarding the use of synergism to screen the initial records "Using these terms may have truncated the search since many authors that report mixture studies do not always classify the interactions using these terms since the study design and data may be insufficient for this classification."

RESPONSE: *ATSDR agrees with this possible explanation of why many interaction studies were apparently not included in the initial literature searches. To counteract this deficiency, ATSDR conducted targeted supplemental literature searches and amended the text in Chapter 2, as described in Responses to Comments 4 and 5 from this Reviewer and amended the text in Chapter 2.*

COMMENT 31: Referring to the statement in the Introduction regarding the search criteria—110 records were selected for retrieval and full text evaluation—the Reviewer made the following comment "Inclusion and exclusion criteria are not clearly stated."

RESPONSE: *Text was amended in Chapter 1 to more clearly state inclusion and exclusion criteria. See responses to Comment 4.*

COMMENT 32: Referring to the statement in the Introduction regarding health guidance values—The critical endpoints for the values identified for the selected metallic cations, based on the most sensitive adverse health effects…—the Reviewer commented "This approach is not clear. Relying on the most sensitive endpoint could be appropriate; however, it ignores metal-metal interactions. For example, a hypothetical case for two metals may be that immunotoxicity is not the most sensitive endpoint; however available studies may show that for this endpoint synergism (or another interaction) is identified. This could make this endpoint more important in a mixture. How did ATSDR consider this?"

RESPONSE: *Based on this comment, the Reviewer appears to be concerned that the literature search and the inclusion and exclusion criteria to select interaction data for the metals of concern were restricted to the critical effects for the health guidance values. They were not. Text and tables in Chapter 1 were modified to make this more clear (for example, see revised Table 2). Appendices A–G describe ATSDR's efforts to develop TTDs for effects known to be associated with oral exposures at doses higher than those associated with the critical effects for the health guidance values (e.g., MRLs). TTDs are the process by which ATSDR's recommended component-based hazard index attempts to consider all effects from a chemical. Unfortunately, available data were inadequate to derive TTDs for most of the*

subject metallic cations, except for barium and magnesium. This result is pointed out in revised Appendices A–G, in all 21 subsections of Chapter 2, and in Chapter 3, including the data needs section.

The major health endpoints identified for repeated oral exposure to the individual metallic cations are shown in Table 2 (see Introduction). The toxicity target of the most sensitive critical effect used to derive health guidance values for repeated oral exposure (MRLs, RfDs, or ULs) is different for each metallic cation in the mixture of concern, with the exception of the kidney, which is the most sensitive target for barium and calcium, and the gastrointestinal tract, which is the most sensitive target for iron and magnesium. Available data from sources used to prepare Appendices A–G were adequate to derive only a few repeated oral exposure TTDs for effects occurring at doses higher than those associated with the respective critical effects for MRLs, RfDs or ULs: TTDs for cardiovascular and neurological effects from barium exposure (Appendix A), kidney effects from magnesium exposure (Appendix D), and reproductive effects from manganese exposure (Appendix E).

COMMENT 33: Referring to the statement in the Introduction—Common targets from repeated oral exposure are the cardiovascular system for sodium and barium, the nervous system for manganese and barium…—the Reviewer made the comment "This is unclear – for example both Mn and Fe can produce extrapyramidal effects thus the nervous system is also a target for Fe. The GIT effects of iron are often more important for high dose exposures versus effects on hematopoetic system, CNS, etc with chronic exposures. How was Table 3 'created' – what criteria were used to make this determination?"

RESPONSE: *Revisions were made to Chapter 1 text and Table 2 explaining how Table 2 was "created;" specifically that it was created from information in sources used to prepare Appendices A–G. Also, changes in the text of Chapters 1, 2, and 3 and Appendix C were made with respect to more accurately discussing the effects associated with iron overload, including neurological effects.*

Chapter 1:

Based on the reviews in the ATSDR or NAS documents deriving the health guidance values, some biological systems are adversely affected by repeated exposure to more than one of the subject metallic cations (see Appendices A–G). Table 2 (prepared from information in Appendices A–G) describes common targets from repeated oral exposure (not necessarily the critical effects) as the cardiovascular system for barium, sodium, and potentially iron, the nervous system for barium, iron, and manganese, the gastrointestinal system for barium, iron, and magnesium, and the kidney for barium, calcium, magnesium, and potentially iron. Additional information on these toxic effects, as well as toxicokinetic and mechanistic considerations, are presented in Appendices A–G. In addition, Toxicity Target Doses (TTDs) for less sensitive effects (i.e., effects occurring at doses higher than those associated with the critical effect for the health guidance value) were derived, if available dose-response data in the review documents were sufficient for derivations. Individuals at risk for primary or secondary iron overload due to altered iron absorption/uptake/excretion because of genetic or disease factors or extremely high oral intakes may develop systemic toxicity involving many organs, particularly the liver and heart, but available dose-response data were insufficient to derive TTDs for these high-dose iron effects (see Appendix C). Information included in the appendices is intended to provide public health assessors with a broad, accurate overview of the toxicity of individual compounds, and is based primarily on existing ATSDR Toxicological Profiles with supplementation information from other public health, occupational health, or regulatory agency guidance documents (e.g., NAS, European Food Safety Authority [EFSA], IRIS, American Conference of Governmental Industrial Hygienists [ACGIH]). In instances where guidance documents were limited to older NAS documents (sodium, iron) and/or specific information was not included in available public health guidance documents (e.g., mechanisms of toxicity), reviews and meta-analyses from the

peer-reviewed literature were included as appropriate. Appendices are not intended to provide a comprehensive review of the primary literature. Primary literature reviewed for this document was limited to studies focusing on possible interactions among the subject metallic cations.

Table 2 footnotes:

[a]Critical effects for derivation of health guidance values (MRLs, RfDs, or ULs) are bolded and italicized. Additional information on other health effects associated with higher exposures to the subject metallic ions is presented in Appendices A-G. Available data were adequate to derive TTDs for cardiovascular and neurological effects from repeated oral exposure to barium (Appendix A). For iron, data were inadequate to derive a TTD for neurotoxicity. For magnesium, data were adequate to derive a TTD for kidney effects (Appendix D). For manganese, data were adequate to derive a TTD for reproductive effects, but the resultant value was equivalent to the RfD. For the other cations, data were inadequate to derive oral TTDs for less sensitive effects occurring above the critical effect for the MRL, RfD, or UL (see Appendices A–G).

[b]Individuals susceptible to primary or secondary iron overload due to genetic disorder/polymorphism or disease state can develop systemic iron toxicity (cardiovascular, neurological, kidney, liver, or reproductive); limited evidence exists for iron overload symptoms in some populations with unusually high dietary intakes in food or drinking water. Available dose-response data were inadequate to derive TTDs for high-dose effects from iron (Appendix C).

[c]ATSDR (2012) did not derive a chronic or intermediate-duration oral MRL for manganese. The RfD for manganese (0.14 mg/kg/day) is recommended for ATSDR public health assessments in the absence of a chronic oral MRL. The RfD is similar in value to the NAS UL for manganese (0.16 mg/kg/day) and is similarly based on a lack of adverse effects associated with average manganese intake levels in Western diets (Appendix E).

COMMENT 34: Referring to the comment—it is unclear if iron overload can occur from increased oral intake in healthy individuals—the Reviewer made the comment "It can occur but this requires high dose exposures (mg/kg). If exposure was a consideration (i.e., ATSDR was focused on 'low dose' responses associated with metal concentrations found in contaminated water) then this should be explicitly stated."

RESPONSE: *ATSDR agrees that the statement was inaccurate. The statement was removed from Chapter 1 and Appendix C text, Table 2, and other places in the document and replaced with more accurate discussions of iron overload effects.*

Legend to Table 2:

[b]Individuals susceptible to primary or secondary iron overload due to genetic disorder/polymorphism or disease state can develop systemic iron toxicity (cardiovascular, neurological, kidney, liver, or reproductive); limited evidence exists for iron overload symptoms in some populations with unusually high dietary intakes in food or drinking water. Available dose-response data were inadequate to derive TTDs for high-dose effects from iron (Appendix C).

Appendix C.2:

Evidence is limited for iron overload symptoms in some populations with unusually high dietary intakes in food or drinking water.

COMMENT 35: Referring to Table 2, the Reviewer commented "Are these really known targets? For example, high intake of supplemental calcium is associated in some studies with an excess risk of CVD death in men but not in women (Xiao 2013). There are similar gaps in this table for other cations – its not clear how this table was generated. For example, magnesium is a risk factor for renal disease. Iron may play a role in some neurodegenerative diseases (e.g., Dusek 2015). It's not clear why the term 'overload' is used in this Table with iron – 'overload' could also apply to manganese (and other essential nutrients) where homeostatic mechanisms exist. Iron poisoning is a well-known concern especially in pediatric cases from multivitamin ingestion (Yuen and Becker, 2018)."

RESPONSE: *See Responses to Comments 1 and 33 concerning changes made on how Table e was created and changes made to iron overload discussions in Chapter 1, Appendix C text, Table 2, and other applicable places in the revised document.*

Appendix B (text already discussed the limited evidence for other effects in humans (including calcium-alkali syndrome, vascular calcification, cardiovascular disease, and prostate cancer) from calcium dietary supplements; these possible effects were not included in Table 2 because the available data were inadequate for UL derivation, and thus for TTD derivation.

Appendix D, Section D.5 was revised to include discussion of evidence for renal effects from high-dose exposure in laboratory animals and the use of these data to derive a kidney TTD for magnesium.

Appendix D, Section D.5 Derivation of Target-organ Toxicity Dose(s):

Following oral exposure, the most sensitive adverse effect associated with excess magnesium is gastrointestinal discomfort and diarrhea, as determined by NAS (1997) and EFSA (2006). A provisional oral MRL of 5 mg Mg/kg/day for intermediate- and chronic-duration exposure of adults was based on the NAS (1997) UL for adults, which was based on an estimated NOAEL of 350 mg/day and a LOAEL of 360 mg Mg/day for mild diarrhea in adults and an uncertainty factor slightly larger than 1.0.

Limited evidence also exists for associations between high acute doses of magnesium sulfate and neurological effects. Although associations between ranges of serum levels of magnesium and neurological symptoms of varying severity have been presented for humans (e.g., Huey et al. 1995), quantitative data relating acute oral doses and neurological symptoms in humans are not available. Thus, a TTD for neurological effects from acute oral exposure to magnesium was not derived.

Statistically significant increased incidences of histological changes in the kidney were found in B6C3F1 mice fed 5% magnesium chloride in the diet (1.362 mg Mg/kg/day), but not in mice fed 2.5% in the diet (647 mg Mg/kg/day) (Tanaka et al. 1994). These kidney effects, or histological changes in other tissues, were not found in F344 rats fed up to 5% magnesium chloride in the diet for 13 week (Takizawa et al. 2000) or in B6C3F1 mice fed supplementary magnesium chloride in the diet for 104 weeks at doses as high as 470 mg Mg/kg/day (Kurata et al. 1989). Following ATSDR MRL derivation protocols, a provisional intermediate-duration TTD for kidney effects of 6 mg Mg/kg/day is derived by dividing the NOAEL, 647 mg Mg/kg/day, for kidney effects in the 13-week mouse study by (Tanaka et al. 1994) by an uncertainty factor of 100 (10 for interspecies extrapolation and 10 for human variation in susceptibility). The value for this provisional intermediate-duration oral TTD for kidney effects from magnesium compounds, 6 mg Mg/kg/day, is slightly higher that the provisional surrogate intermediate-duration MRL of 5 mg Mg/kg/day, based on the NAS (1997) UL of 350 mg Mg/day for mild diarrhea in adults.

COMMENT 36: Referring to the statement in the section on iron and manganese—The influence of excess manganese on iron homeostasis may be dose-, route-, or media- dependent..—the Reviewer made the comment “Will also likely be age dependent since biliary excretion and homeostatic mechanisms are incompletely developed in neonates.”

RESPONSE: *The referenced statement in Section 2.2.13 was revised to include age:*

The influence of excess manganese on iron homeostasis may be dose-, route-, age-, or media-dependent, as suggested by the observation that exposure of nutritionally iron-sufficient rats to drinking water with 10 mg Mn/mL had no influence on levels of iron in tissues from three brain

> regions (globus pallidus, striatum, and inferior colliculus) or three regions of the cochlea, compared with controls without added manganese in drinking water (Mullin et al. 2015).

It is noted that Section 2.2.13 contains examples of age and developmental stage influence of excess manganese on iron homeostasis.

COMMENT 37: Referring to the statement in the section on iron and manganese —Differences between animal species or stage of development also may influence potential interactions between iron and manganese—the Reviewer commented "See earlier comment this paragraph." This is referring to COMMENT 36.

RESPONSE: *See Response to Comment 36.*

COMMENT 38: Referring to the statement in the section on iron and manganese—There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome or adverse effects in a common target organ…—the Reviewer made the comment "I'm not sure I understand this. For example, both Mn and Fe are thought to enhance ROS production – albeit Fe is more 'potent' since it catalyzes Fenton reactions. Also both accumulate in the human substantia nigra and produce similar clinical effects (Liu et al., 2017). This statement seems to need a bit more explanation."

RESPONSE: *The sentence (here and in the other 20 subsections of Chapter 2) was missing an important phrase that was inadvertently deleted in document preparation. In revising the document, it was replaced with:*

> There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome via a common mode of action or adverse effects in a common target organ or tissue.

Also, revisions were made in Section 2.2 (and Chapter 3) to highlight evidence that: (1) effects from excess iron and excess manganese can occur, at least in part, via a common mode of action (reactive oxygen species) and (2) neurological effects from both metallic cations are possible (see new paragraph on this topic in revised Section 2.2.13).

Section 2.2.13:

> The nervous system may be a common toxicity target of excess iron and excess manganese, as evidenced by observations of: (1) neurological dysfunction in human subjects with genetic disorders or neurodegenerative diseases such as Parkinson's and Alzheimer's disease and accumulation of iron, manganese, and other metals in brain tissue (Chen et al. 2019; Dusek et al. 2015a, 2015b; Huat et al. 2019); (2) neurological dysfunction and accumulation of manganese in brains of human subjects with high occupational exposures to manganese (e.g., manganese miners, see ATSDR 2012); and (3) common proposed toxic actions related to generation of reactive oxygen species (see Appendices C and E).

Section 3.2:

> Available evidence suggests that: (1) iron-deficiency or fortification in laboratory animals may enhance the neurotoxic effects of manganese via enhanced distribution of manganese to the brain (Section 2.2.13); (2) brain accumulation of iron and manganese and other metals is associated with neurodegenerative diseases, indicative of possible joint neurotoxic actions of iron, manganese, and other metals involving neuronal damage from reactive oxygen species

(Section 2.2.13); (3) dietary calcium supplementation may be without effect on manganese balance in human balance studies (Section 2.2.9); and (4) magnesium at relatively high dietary levels (compared with manganese levels) inhibited short-term gastrointestinal absorption of manganese in mice and prevented deaths in pigs fed diets with inadequate magnesium levels and high levels of manganese (Section 2.2.16).

COMMENT 39: Referring the statement in the section on iron and manganese—... the principal and critical effects of repeated exposure to excess iron are gastrointestinal discomfort and irritation (see Appendices D and E)—the Reviewer made the comment "What about some of the neurotox studies performed with dietary Mn and Fe? What was the basis to dismiss these (e.g., Fitsanakis et al. 2011)?"

RESPONSE: *The intent was not to dismiss Fitsanakis et al. (2011). The text in Section 2.2.13 was revised to better highlight the results of this study; other changes were made as well to emphasize the evidence that the nervous system represents a likely common toxicity target for both metallic cations.*

Complementing the toxicokinetic evidence, rats fed an iron-deficient and high-manganese diet had increased brain levels of oxygen stress indicators and altered results in a water maze behavioral test, compared with rats fed a control diet with normal levels of iron and manganese (Fitsanakis et al. 2009).

COMMENT 40: The Reviewer made the following comment in the section on manganese and strontium—"Here and elsewhere. It's difficult to judge whether possible mechanisms were considered. For example Mn and Sr and Ba interact with Na,K-ATPase – this potential target may or may not be toxicologically important (Gatto et al., 2007). A significant concern as mentioned earlier is that potential modes of action [vs. organ targets) is not being considered. It may be useful if ATSDR provided the main modes of action – e.g., Sr binds and activates the Calcium-sensing Receptor (CaSr) triggering different osteoblast signaling pathways. Mn, Mn2+ can enter cells via the same transport systems in the same way as Ca2+ and can bind to a number of intracellular structures because of its high affinity for Ca2+ and Mg2+ binding sites. Mn interacts with CaSr as well (e.g., Baio et al 2012) – so is this a possible way Ca, Mn, and Sr might interact?"

RESPONSE: *In response to this and other comments from this Reviewer, supplemental literature searches were conducted (see Response to Comments 4 and 5) to identify studies of interactions among the cations at the cellular level of organization, especially for the pairs of metals identified in the previous draft as data poor. Many studies were located and retrieved and a discussion of the results and their significance to the primary focus of the profile (how do possible interactions influence expression of toxicity) was added to each of the Chapter 2 subsections identified previously as data poor (such as Section 2.2.20):*

Years of research have shown that manganese and strontium and other divalent cations can interact in complex ways with isolated membrane transport systems including: (1) components of mammalian calcium homeostatic systems including several types of calcium channels (see Sections 2.2.9 and 2.2.11); (2) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (3) TRP channels, a diverse family of mammalian cation channel and kinases (see review by Bouron et al. 2015); (4) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et al. 2012); and (5) calcium-activated SK potassium channels expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). Studied points of divalent cation interaction with these transport proteins include inhibition of, and competitive permeation through, the ion conducting channels and modulation of activity through binding sites that cause conformational changes in protein three-dimensional structure.

The study of these interactions of divalent cations on transport proteins has been important to understanding how the systems work in isolation, but the physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with regard to making reliable predictions of how combined oral exposure to any pair of metallic cations that are the subject of this profile may influence their individual toxicity.

COMMENT 41: Referring to the statement in the section on barium and manganese—… neurological effects have been reported with higher barium exposures, indicating a common toxicity target of manganese and barium—the Reviewer commented "Neurotoxicity with barium is a high dose phenomenon (recall its used as a GIT radiocontrast agent in people) – this again raises the concern about Tables in Chapter 1 being overly selective about endpoints. It may also be worth mentioning that Ba effects may be secondary to effects on K (and/or direct) and Mn and B do not share common neurologic targets."

RESPONSE*: See Responses to Comments 4 and 5 about revisions to Tables in Chapter 1. Revised Section 2.2.4 discusses that barium and manganese neurological effects are caused by different modes of action. ATSDR thinks that the document clearly notes (many places, including Chapter 1, Chapter 2 subsections involving barium, Chapter 3, and Appendix A) that neurotoxicity of barium occurs at doses above the critical kidney effects used as the basis of the health guidance value (the MRL) and thinks that the principal target audience, risk assessors and public health assessors, will understand the value of TTDs.*

COMMENT 42: Referring to the statement in the summary section on calcium and manganese—Although there is evidence for complex interactions between short-term exposures to manganese and calcium at subcellular, cellular, tissue and whole-body levels of organization, consistent evidence for marked negative manganese balance in the presence of calcium dietary supplements has not been provided by human balance studies—the Reviewer comments "I don't disagree but the document is not clear as to whether it is evaluating hazards or dose-response relationships/risk. For some binary mixtures dose is not really considered in a meaningful way (e.g., Mn and Ba above) so the way weight of evidence is being applied is at best inconsistent."

RESPONSE: *In subsections of Chapter 2, data about dose-dependence of possible interactions between pairs of the cations were discussed if they were available for* in vivo *oral exposure, as was the case for Section 2.2.9, Calcium and Manganese. For many binary mixtures, however, whole-body interaction data were not available (such as for Section 2.2.4, Barium and Manganese and Section 2.2.20, Manganese and Strontium) and dose-dependence of this level of interaction could not be discussed. Each subsection that was identified as data poor in the previous version was revised with cellular interaction data; however, ATSDR did not provide details about dose-dependence of interactions because the toxicological significance of a specific point of cellular-level interaction is unclear for the essential metals with many points of interaction. ATSDR thinks that this is a logical approach and has applied it consistently across the 21 subsections of Chapter 2. See examples below from revised Sections 2.2.4 and 2.2.20. This approach is also consistent with the weight-of-evidence approach used in this assessment, which does not include dose in the BINWOE derivation (Mumtaz and Durkin 1992).*

Section 2.2.4:

Barium and manganese and other divalent cations can interact in complex ways with isolated membrane transport systems including: (1) components of mammalian calcium homeostatic systems including several types of calcium channels (see Sections 2.2.1 and 2.2.9); (2) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (3) TRP channels (Bouron et al. 2015);

(4) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et al. 2012); and (5) calcium-activated SK potassium channels expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). Also, barium, manganese, and nickel, like calcium, have been shown to activate purified nitric oxide synthase, a Ca^{+2}/calmodulin binding enzyme that mediates production of nitric oxide free radicals, which are thought to be involved with vasodilator tone, hypertension, and neuronal function (Weaver et al. 2004). The physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with regard to making reliable predictions of how repeated combined oral exposure to any pair of metallic cations that are the subject of this profile may influence their individual toxicity.

No studies were located that examined toxicokinetic, nutritional balance, or toxicological endpoints following repeated oral exposure of humans or laboratory animals to concomitant excess levels of barium and manganese; these types of studies would be more useful for understanding how repeated oral exposure to excess levels of both barium and manganese may influence each other's toxicity. In deriving health guidance values to protect against the development of adverse effects from repeated oral exposure, the critical effects from exposure to manganese are neurological, whereas the critical effects from exposure to barium are kidney effects (see Appendices A and E). However, neurological effects have been reported with higher barium exposures and kidney effects at lower exposures, indicating a common toxicity target of barium and manganese, albeit by separate modes of action (see Appendices A and E). Because dose-response data for barium-induced neurological effects are available, a TTD was derived for neurological effects following repeat oral exposure to barium (see Appendix A).

Summary. Studies conducted in subcellular systems, isolated cells, and tissues have found evidence for various interactions between barium and manganese on membrane transport of ions and neurotransmitters and on nitric acid synthetase. However, there is inadequate evidence to make conclusions on whether or not repeated oral exposure to excess barium and manganese may influence each other's toxicity or whether their possible joint actions on the nervous system are additive, less-than-additive, or greater-than-additive.

Section 2.2.20:

Years of research have shown that manganese and strontium and other divalent cations can interact in complex ways with isolated membrane transport systems including: (1) components of mammalian calcium homeostatic systems including several types of calcium channels (see Sections 2.2.9 and 2.2.11); (2) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (3) TRP channels, a diverse family of mammalian cation channel and kinases (see review by Bouron et al. 2015); (4) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et al. 2012); and (5) calcium-activated SK potassium channels expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). Studied points of divalent cation interaction with these transport proteins include inhibition of, and competitive permeation through, the ion conducting channels and modulation of activity through binding sites that cause conformational changes in protein three-dimensional structure. The study of these interactions of divalent cations on transport proteins has been important to understanding how the systems work in isolation, but the physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with regard to making reliable predictions of how combined oral exposure to any pair of metallic cations that are the subject of this profile may influence their individual toxicity.

No studies were located that examined toxicokinetic, nutritional balance, or toxicological endpoints following repeated oral exposure of humans or laboratory animals to concomitant excess levels of manganese and strontium; these types of studies would be more useful for understanding how repeated oral exposure to excess levels of both may influence each other's toxicity. There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome via a common mode of action or adverse effects in a common target organ or tissue (see Appendices E and G). In deriving health guidance values to protect against the development of adverse effects from repeated oral exposure, the critical adverse effects observed in humans and laboratory animals excessively exposed to manganese are neurological, whereas the critical effect of repeated exposure to excess strontium is skeletal effects (see Appendices E and G). Overlapping toxicity targets across a range of exposure conditions have not been clearly identified and TTDs for other less sensitive effects were not developed for manganese and strontium due to inadequate data (Appendices E and G).

Summary. Studies conducted in subcellular systems, isolated cells, and tissues have found evidence for various interactions between manganese and strontium on membrane transport of ions and neurotransmitters. However, there is inadequate evidence to make conclusions whether or not repeated oral exposure to excess manganese and strontium may influence each other's toxicity.

COMMENT 43: Referring to the statement in the section on calcium and manganese—the principal and critical effect of repeated exposure to excess calcium is increased risk for kidney stones—the Reviewer made the comment "What about skeletal effects?"

RESPONSE: *Appendix B discussed the selection of kidney effects as the critical effect for excess calcium among other candidate choices (see Response to Comment 35). Adverse skeletal effects are well known as a hazard from calcium deficiency, but do not appear to be of concern from supplemental calcium intake.*

COMMENT 44: The Reviewer made the following comment in magnesium and manganese— "General comment that goes beyond this binary interaction. It can be very challenging to assess metal-metal interactions in animal studies since the levels of many essential nutrients in lab chow diets are often heavily supplemented and far exceed NRC recommendations (for multiple nutrients). This point should be acknowledged somewhere in the document since they may not mimic mineral intakes in people."

RESPONSE: *A statement about this challenge was added to Chapter 3:*

For this document, data on potential interactions between pairs of the selected metallic cations were identified and evaluated to assess evidence that could qualitatively modify public health assessments (using component-based hazard index approaches) for mixtures with the metallic cations of concern. A summary of this analysis (presented in Chapter 2) follows. This binary approach is acknowledged to be a practical approach with inherent uncertainty due to evidence that coupling of metallic cation homeostatic mechanisms is complex and can overlap for two or more metals. As such, changes in tissue distribution of metals, and subsequent toxic responses, seen with co-exposure to two metals may not be the same as those found with simultaneous exposure to the same two metals plus additional metals (see Section 2.1). Another area of uncertainty is that metal-metal interactions observed in laboratory animal studies may not be directly applicable to humans because diets for laboratory animals may often be more heavily supplemented with essential metals than human diets.

COMMENT 45: Referring to the statement in the section on sodium and strontium—No data were located regarding potential interactions between sodium and strontium in biological systems—the Reviewer commented "There are some studies in birds fed different levels of Sr and then Na concentrations evaluated – with different vitamin D levels in the diet (e.g., see: Browning & Cowieson 2015). Again it's not clear if these types of studies were not found or excluded."

RESPONSE: *This type of study examining the influence of exposure to one metal on tissue distribution or retention of other metals provides suggestive evidence for putative interactions at the whole-body level of organization (in this case, supplemental strontium exposure causes differences in tissue retention of sodium dependent on vitamin D status), but it does not inform how co-exposure to both cations may influence tissue distribution or retention and toxicity expression. Thus, such studies are not directly useful to the main evaluations in the document and were not specifically selected.*

Section 2.2.21 was revised (as were other "data poor" Chapter 2 subsections) to discuss evidence for interactions at the cellular level. In addition, discussion of results of the Browning and Cowieson (2015) study was added to Section 2.2.21 with the statement that the relevance to scenarios of co-exposure to excess sodium and strontium is unclear:

> Evidence for complex interactions between sodium and strontium have been demonstrated in a study that examined retention of sodium, phosphorus, and strontium in chickens provided varying levels of strontium and vitamin D3 (D3, Browning and Cowieson 2015). The results showed that supplemental strontium (500 or 1,000 mg Sr/kg diet) increased phosphorus, sodium, and strontium retention in birds fed 2,500 IU D3/kg diet kg, but reduced phosphorus, sodium, and strontium retention in birds fed 5,000 IU D3/kg diet. Although these results are consistent with strontium influencing the whole-body retention of sodium, their relevance to scenarios of co-exposure to excess sodium and strontium is unclear.

COMMENT 46: Referring to the statement in the section on sodium and strontium—whereas the principal and critical effects of repeated exposure to excess strontium are skeletal—the Reviewer made the comment "Sodium has effects on bone density e.g., Heaney 2006; others)."

RESPONSE: *Appendix F discusses the evidence that high sodium salt intake has been associated with skeletal effects (osteoporosis), and that available dose-response data were inadequate for TTD development. The summary for Section 2.2.21 was revised to discuss this limitation:*

> No studies were located that examined toxicokinetic, nutritional balance, or toxicological endpoints following repeated oral exposure of humans or laboratory animals to concomitant excess levels of sodium and strontium; these types of studies would be more useful for understanding how repeated oral exposure to excess levels of both may influence each other's toxicity. There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome via a common mode of action, but skeletal effects have been associated with high sodium salt intake (osteoporosis; Appendix F) and excess strontium intake (skeletal abnormalities in children with poor diets (vitamin D, calcium, and protein deficiencies; Appendix G). In deriving health guidance values to protect against the development of adverse effects from repeated oral exposure, the critical adverse effect for exposure to sodium is hypertension, whereas the critical effects of repeated exposure to strontium are skeletal effects (see Appendices F and G). TTDs for skeletal effects from high sodium salt intakes and for other less sensitive effects from these metallic cations alone were not developed due to inadequate data (see Appendices F and G).

Summary. Studies conducted in subcellular systems, isolated cells, and tissues have found evidence for various interactions between sodium and strontium on membrane transport. However, the available interaction data are inadequate to conclude whether or not repeated concomitant oral exposure to sodium and strontium will influence each other's critical toxic effects. Additional studies of the effects of co-exposure to excess sodium and strontium on skeletal endpoints could provide better information on how they may jointly act to produce adverse skeletal effects.

COMMENT 47: Referring to the statement in the section on barium and sodium—No data were located regarding potential interactions between sodium and barium in biological systems—the Reviewer made the comment "What about Perry et al., 1989 that looked at Ba and cardiovascular effects and then measured tissue calcium, magnesium, sodium, or potassium?"

RESPONSE: *Discussion of Perry et al. (1989) was not added to Section 2.2.5; see Response to Comment 45 regarding limited relevance of this type of study examining the influence of exposure to one metal on tissue distribution or retention of other metals.*

However, other comments (4, 5, 40, 41, and 42) from this Reviewer led to significant revisions to Section 2.2.5, including evidence for barium and sodium interactions from studies of membrane transport in isolated cells and in vivo *evidence for barium inhibition of sodium resorption in rat kidneys due to barium blockage of potassium channels and subsequent disruption of the operation of a $Na^+/K^+/Cl^-$ co-transporter (Huang et al. 2000; Walter et al. 2001; Wang et al. 1995).*

COMMENT 48: The Reviewer made the following comment in the section on magnesium and sodium—"Some transporters for Mg have been identified in patients with familial hypomagnesemia (Scheinman 2014; others). As mentioned earlier there could also be effects on the kidney where interactions in the Loop of Henle could occur. Not clear why effects on blood pressure were the sole endpoint of interest here."

RESPONSE: *Section 2.2.17 was revised: (1) to expand and update discussion about sodium/magnesium exchange in cellular homeostasis for these metallic cations and (2) to discuss* in vivo *studies showing effects of high sodium salt intake on renal handling of both magnesium and calcium and indicating coupling among several homeostatic mechanisms for sodium, magnesium, and calcium.*

Sodium/magnesium exchange across cellular membranes (sodium influx with magnesium efflux) has long been thought to be an integral part of homeostatic regulation of cellular magnesium. For example, early studies with mammalian cells showed that substitution of extracellular sodium ion by choline inhibited magnesium efflux indicating that magnesium efflux is sodium dependent (Günther et al. 1984). Currently, however, the identity of the membrane transport proteins responsible for the sodium/magnesium exchange is somewhat controversial. Funato et al. (2018) reviewed evidence that linked dysfunctional CNNM2, a divalent metal cation transporter, to defects in renal reabsorption in mice and zebrafish, and presented evidence that this membrane functions as a sodium/magnesium exchanger. Arjona and de Baaij (2018) presented evidence for an alternative hypothesis that CNNM2 is not the sodium/magnesium exchanger, but rather regulates magnesium influx via TRPM6 and TRPM7 and indirectly affects an independent exchange of magnesium efflux and sodium influx. In a third viewpoint, Kolisek et al. (2019) presented evidence that SLC41A3 plays a role in sodium/magnesium exchange across cellular membranes. Others have provided evidence with isolated cells that the electrogenic $Na+$-HCO_3^- cotransporter, NBCe1-B, which is involved in regulation of intracellular pH and epithelial HCO_3^- secretion, is regulated by intracellular magnesium concentrations, suggesting another coupling

site between magnesium and sodium homeostasis (Yamaguchi and Ishikawa 2008). Regardless of the uncertainty in the molecular details, the available evidence from these types of investigations suggests that there may be coupling between magnesium and magnesium homeostatic mechanisms in at least two cellular sites but this does not explain how repeated oral co-exposure to excess magnesium and sodium may influence each other's toxicity.

In rodent studies, high sodium salt oral intake by rats for 1 week was associated with increased urinary excretion of sodium, calcium, and magnesium along with upregulation of genes for transport proteins for calcium (TRPV5, TRPV6, calbindin-D28K) and magnesium (TRPM6) in the renal distal convoluted tubule (Lee et al. 2012), whereas in another study, high sodium salt oral intake (with normal or low potassium intake) by mice for 4 days was associated with increased urinary excretion of calcium and sodium, but not magnesium, along with renal upregulation of magnesium transporter TRPM6 and calbindin-D28K, down regulation of magnesium transporter TRPM7, and no change in expression of renal genes for calcium transporter TRPV5 (van der Wijst et al. 2018). The apparent differences in the urinary excretion and renal gene expression profiles in the two studies may be due to differences in duration of exposure to the high salt conditions (7 versus 3 days), suggesting time-dependent responses, but differences in species and salt administration (drinking water vs diet) or some other factor could also be involved. Together, however, the results demonstrate effects of high sodium salt intake on renal handling of both calcium and magnesium and indicate coupling among several homeostatic mechanisms for calcium, magnesium, and sodium.

COMMENT 49: Referring to the statement in the section on barium and strontium—There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome or adverse effects in a common target organ or tissue—the Reviewer made the comment "They share some common cellular/enzymatic targets – some of which have been mentioned earlier."

RESPONSE: *Section 2.2.6 was revised to discuss interactions among strontium, barium, and calcium, with membrane transport systems in isolated cells or tissues.*

Barium, calcium, and strontium belong to Group (IIA) of the periodic table of elements. These elements have the same number of valence electrons and form stable divalent cations. The Stokes radius of Ba^{2+} is approximately 6% smaller than Ca^{2+} and Sr^{2+} (Kadhim and Gamaj 2020). These characteristics influence their interactions with binding sites on proteins, and extensive research has focused on their interactions with isolated membrane transport systems including: (1) components of mammalian calcium homeostatic systems including several types of calcium channels (see Sections 2.2.1 and 2.2.11); (2) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (3) TRP channels (Bouron et al. 2015); (4) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et al. 2012); and (5) calcium-activated SK potassium expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). Findings from these studies have been important to understanding how the systems work in isolation, but the whole-body physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with respect to making reliable predictions of how combined oral exposure of humans or laboratory animals to any pair of metallic cations that are the subject of this profile may influence each other's toxicity.

COMMENT 50: Referring the statement in the section on calcium and strontium—Whether or not the joint action of these cations on osteoclast apoptosis is dose-additive…—the Reviewer made the comment

"Also possible interactions via AMPK/mTOR signaling pathway (e.g., see Zhang et al., 2017; Cheng et al., 2019) that may contribute to skeletal effects."

RESPONSE: *The discussion of the beneficial effects of strontium in Section 2.2.11 in improving bone formation and inhibiting bone resorption was amended to add evidence that strontium stimulation of AMPK/mTOR signaling is involved.*

> Strontium stimulation of the AMP-protein kinase and mammalian target of rapamycin (AMPK/mTOR) signaling pathway has been linked to strontium stimulation of autophagy and differentiation in MC3T3 osteoblastic cells (Cheng et al. 2019). Whether or not the joint action of these cations on osteoclast apoptosis or differentiation is dose-additive, greater-than-dose-additive, or less-than-dose-additive has not been determined.

COMMENT 51: Referring to the discussion on interactions with CaSR in the section on calcium and strontium, the Reviewer made the comment "See earlier comment that Mn (and possibly other cations) also have some interactions with CaSR)."

RESPONSE: *No changes to Section 2.2.1 (Strontium and Calcium) were necessary in response to this comment. Interactions between calcium and manganese through magnesium interactions with CaSR are discussed in Section 2.2.9.*

COMMENT 52: Referring to the statement in the section on calcium and manganese—… excess exposure to either of these metallic ions alone results in a common adverse outcome or adverse effects in a common target organ or tissue—the Reviewer made the comment "Skeleton? Why is this not considered a common target organ?"

RESPONSE: *As previously noted in the Response to Comment 43, Appendix B discusses the selection of kidney effects as the critical effect for excess calcium among other candidate choices. Adverse skeletal effects are well known as a hazard from calcium deficiency, but do not appear to be of concern from supplemental calcium intake.*

COMMENT 53: Referring to the statement in the section on magnesium and strontium—No data were located regarding potential interactions between strontium and magnesium in biological systems—the Reviewer made the comment "Both interact with Ca transport systems (e.g., Janda 1969; others)"

RESPONSE: *Section 2.2.18 was revised to include a discussion of interactions of strontium and magnesium with elements of calcium homeostasis (e.g., calcium channels and calcium-binding regulatory proteins) and interactions of strontium and magnesium with other isolated transport systems like sodium pumps and TRP kinase channels.*

> Interactions with elements of calcium homeostasis (e.g., calcium channels and calcium-binding regulatory proteins) in isolated cells have been extensively studied with magnesium (Section 2.2.8) and strontium (see Section 2.2.11), owing, at least in part, to shared membership in Group IIA of the periodic table of elements. Both form stable divalent cations. The stokes radii of Ca^{2+} and Sr^{2+} are nearly identical; the Stokes radius of Mg^{2+} is approximately 12% larger than Ca^{2+} and Sr^{2+} (Kadhim and Gamaj 2020). Many studies have also examined interactions of magnesium and strontium with other isolated transport systems including: (1) sodium pumps (Cukierman and Krueger 1990; Gatto et al. 2007); (2) TRP channels (Bouron et al. 2015); (3) calcium-activated BK potassium channels involved in the regulation of neurotransmitter release and neuronal excitability (e.g., Lee and Cui 2010; McLarnon and Sawyer 1993; Zhou et

al. 2012); and (4) calcium-activated SK potassium expressed in neurons, smooth muscle, neuroendocrine cells, and hematopoietic cells (e.g., Cao and Houamed 1999). The physiological and toxicological relevance of the findings from this type of research is mostly unclear, especially with regard to making reliable predictions of how combined oral exposure to excess magnesium and strontium may influence each other's toxicity.

COMMENT 54: Referring to the statement in Section 3.1—The exposure scenario of greatest concern for this mixture is chronic-duration, low-level oral exposure to contaminated drinking water—the Reviewer made the comment "This is an important point that could be made earlier."

RESPONSE: *Revised text in Chapter 1 justifies and emphasizes this focus.*

Thus, the exposure scenario of greatest concern for this mixture and interaction profile is chronic-duration, environmentally relevant oral exposure to contaminated drinking water. Environmentally relevant concentrations of the subject cations in groundwater and drinking water wells are expected to be lower than concentrations detected in UOG extraction waste water samples (see Appendix I), due to dilution and ground filtering. Noncancer effects from these cations were the focus of this profile because none of them have cancer weight-of-evidence determinations or cancer slope factors, which would be used in public health assessments (see Appendices A–G).

COMMENT 55: Referring to the statement in Section 3.1—ATSDR recommends a component-based hazard index approach assuming additivity for preliminary screening-level public health assessment, with no grouping based on common adverse outcome or common mode of action—the Reviewer made the comment "This could be reasonable – however, mode of action has been a driver for cumulative risk assessments for other classes of agents. The draft document is inconsistent in considering mode of action."

RESPONSE: *The revised Chapter 2 discusses interactions at the cellular level more extensively and uniformly among the pairs of metallic cations (see responses to Comments 5 and 9). Mode of action is considered when evidence is available to suggest a commonality; among the subject metals, manganese and iron have the best evidence for a shared mode of action (i.e., tissue damage from reactive oxygen species).*

COMMENT 56: The Reviewer made the following comment in Section 3.1 "The document would be strengthened here if formulas to derive a hazard index and hazard quotient are provided."

RESPONSE: *The formula for the mixtures Hazard Index is provided in Section 3.1. The text was revised to refer the reader to the formulas.*

In this approach, the ratios of exposure levels to health guidance values (hazard index) for each substance affecting a particular endpoint (hazard quotients) are summed to provide a measure of hazard for the whole mixture (see formulas for hazard quotients and hazard index below).

COMMENT 57: Referring to the statement in Section 3.1—ATSDR recommends that hazard indexes be calculated if two or more of the individual components have hazard quotients ≥0.1; …—the Reviewer made the comment "Is this science based or a policy decision? How was 0.1 identified as the cutoff?"

RESPONSE: *These are ATSDR policy decisions discussed in ATSDR (2004a and 2018).*

ATSDR. 2004a. Guidance manual for the assessment of joint toxic action of chemical mixtures. Agency for Toxic Substances Disease Registry. http://www.atsdr.cdc.gov/interactionprofiles/IP-ga/ipga.pdf. September 8, 2015.

ATSDR. 2018. Framework for assessing health impacts of multiple chemicals and other stressors (update). Atlanta, GA: Agency for Toxic Substances and Disease Registry. https://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf. December 22, 2020.

COMMENT 58: Referring to the statement in Section 3.1—… individual hazard quotients or the mixture hazard index >1—the Reviewer made the comment "Similar question – is this science based or ATSDR policy? Why 1.0?"

RESPONSE: *These are ATSDR policy decisions discussed in ATSDR (2004a and 2018).*

ATSDR. 2004a. Guidance manual for the assessment of joint toxic action of chemical mixtures. Agency for Toxic Substances Disease Registry. http://www.atsdr.cdc.gov/interactionprofiles/IP-ga/ipga.pdf. September 8, 2015.

ATSDR. 2018. Framework for assessing health impacts of multiple chemicals and other stressors (update). Atlanta, GA: Agency for Toxic Substances and Disease Registry. https://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf. December 22, 2020.

COMMENT 59: The Reviewer made the following comment in Section 3.1 referring to the calculation of hazard index for mixtures "In this approach different toxicity values are considered and UFs are applied to these values. There is some debate as to which UFs to apply (e.g., database uncertainty/modifying factor). This is also combining different toxicity values (e.g., tolerable upper intake level and minimal risk levels without clear justification – other than these values are available. Why not develop a standardized approach? For example, why would the UL for manganese not be used since like iron it is an essential nutrient (the UL would be higher than the RfD)."

RESPONSE: *Revised text in Chapters 1 and 3 more completely describe the approach used for metals without ATSDR oral MRLs. For four essential metals (sodium, iron, calcium, and magnesium), the only option is to use the NAS ULs (no MRLs or RFDs are available). For manganese, the values of the NAS UL and the EPA RfD for manganese are similar and are similarly based on the absence of neurological effects in the general population with normal manganese intakes. ATSDR (2004a, 2018) practice in public health assessments is to use EPA RfDs for chemicals in the absence of an ATSDR MRL.*

ATSDR. 2004a. Guidance manual for the assessment of joint toxic action of chemical mixtures. Agency for Toxic Substances Disease Registry. http://www.atsdr.cdc.gov/interactionprofiles/IP-ga/ipga.pdf. September 8, 2015.

ATSDR. 2018. Framework for assessing health impacts of multiple chemicals and other stressors (update). Atlanta, GA: Agency for Toxic Substances and Disease Registry. https://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf. December 22, 2020.

COMMENT 60: Referring to the statement in Section 3.1—TTDs are developed for an endpoint of concern when the critical effect levels for those effects are higher than those associated with the most sensitive endpoint—the Reviewer made the comment "But ATSDR largely seemed to ignore these less sensitive effects that may indeed be more important from a mixture/cumulative exposure perspective."

RESPONSE: *ATSDR agrees that these high-dose effects are important from a "mixture/cumulative exposure perspective;" that is why ATSDR attempted to make TTDs for each of the seven metallic cations as discussed in Appendices A–G. See Response to Comment 32 about revisions made to clarify that TTD development was attempted but could not be achieved in most cases due to the lack of adequate dose-response data.*

COMMENT 61: Referring to the statement in Section 3.1—…it is unclear if iron overload can occur from increased oral intake in healthy individuals—the Reviewer made the comment "As mentioned earlier I don't understand this statement since iron toxicity occurs frequently in previously healthy individuals. Is ATSDR ignoring the acute [clinical] toxicology literature with iron?"

RESPONSE: *The statement was removed here and elsewhere in the document (e.g., see Response to Comment 33).*

COMMENT 62: Referring to the statement in Section 3.1—toxic effects associated with iron overload (e.g., cardiomyopathy)—the Reviewer made the comment "The ATSDR needs to carefully distinguish what it means by iron overload – I assume you actually mean hereditary hemochromatosis vs. Fe overload that may occur when Fe binding capacity is overwhelmed."

RESPONSE: *Text was revised many places in the document to distinguish between genetically based iron overload and other conditions (such as overwhelming homeostatic mechanisms) leading to high tissue iron accumulation.*

Introduction, Table 2 legend:

> Individuals at risk for primary or secondary iron overload due to altered iron absorption/uptake/excretion because of genetic or diseased factors or extremely high oral intakes may develop systemic toxicity involving many organs, particularly the liver and heart, but available dose-response data were insufficient to derive TTDs for these high-dose iron effects (see Appendix C).

Section 2.2.14 Iron and Sodium:

> High acute oral fatal doses of iron (~60 mg/kg) have been associated with fatality due to iron overload, with involvement of the cardiovascular system, central nervous system, kidney, liver, and hematological systems, and genetically determined chronic iron-overload conditions (e.g., thalassemias, congenital atransferrinemia, and aceruloplasminemia) have been associated with multiple other adverse outcomes in humans, including liver cirrhosis, cardiomyopathy, and neurodegeneration (see Appendix C).

COMMENT 63: The Reviewer made the following comment in Section 3.1 referring to the hazard index formula "See earlier comment regarding mixing toxicity values. This document would be strengthened if the basis for the TTD calculations would be shown in this Chapter (here and elsewhere)."

RESPONSE: *Table 3 presents the TTDs and refers the reader to the appropriate appendices for additional details.*

COMMENT 64: Referring to the following statement in Section 3.1—…with hazard quotients for calcium using the calcium UL and for barium using the barium RfD or MRL)—the Reviewer made the comment "Basis for making this choice?"

RESPONSE: *See Response to Comment 59.*

COMMENT 65: Referring to Table 5, the Reviewer commented "Calcium oxalate uroliths formation is impacted by dietary levels of some of these nutrients – this is well recognized clinically in veterinary species. Again, how was this type of nutritional/toxicity data considered in this assessment?"

RESPONSE: *See also the Response to Comment 15 from this Reviewer. Calcium oxalate urolith formation (calcium kidney stones) in humans is well recognized from human epidemiological studies and in human clinical practice as being impacted by multiple factors, in a similar manner as in veterinary species. Revisions were made to Appendix B Sections B.2 and B.3 to mention some of the literature on veterinary species (cats and dogs), and update and expand on the multiplicity of risk factors involved in calcium oxalate stone formation in humans.*

Section B.2:

Other recent reviews present evidence that kidney stone disease in humans has multiple risk factors including sex, age, race, family history, several dietary factors other than calcium (e.g., higher dietary intakes of animal protein, sugar, or oxalates may increase risk and higher potassium or phytates may decrease risk), systemic disorders (e.g., obesity and diabetes increase risk), and fluid intake (e.g., low fluid intake increases risk) (Curhan 2007; Prochaska et al. 2016). The multiplicity of risk factors reflects the inconsistency among epidemiology studies finding positive associations between calcium intake and kidney stones. A number of prospective epidemiology studies have now consistently found an inverse relationship between dietary calcium and kidney stone risk, but intake of calcium supplements has been found to present a 20% added risk for kidney stones in older women, but not in younger women (Prochaska et al. 2016).

Section B.3:

Other studies of humans (Curhan et al. 1993, 1997, 2004), rodents (Mourad et al. 2006), and cats and dogs (Lekcharoensuk et al. 2001a, 2001b; Lulich et al. 2016) have observed inverse relationships between calcium dietary intakes and kidney stone risk (less risk with higher dietary calcium) and provided evidence that other components of the diet can influence the apparent association between high calcium intakes and formation of kidney stones (e.g., increased intakes of oxalates and sugar increase risk and increased intakes of potassium and phytates may decrease risk). A postulated mechanistic explanation for the apparent discrepancy between dietary calcium and supplemental calcium is that dietary calcium may inhibit oxalate absorption in the gut more effectively than supplementary calcium not taken with meals (Prochaska et al. 2016). Overall, the mechanism of kidney stone formation in humans is not clearly understood, but multiple risk factors other than calcium intake from supplements are widely recognized, including low fluid intake, increased urinary calcium and oxalate excretion, increased dietary sugar, high body mass index, and type II diabetes (Curhan 2007; EFSA 2012; NAS 2011; Prochaska et al. 2016).

COMMENT 66: Referring to the statement in the legend in Table 4)—+ =1–5 studies indicating coupling at one homeostatic mechanism/process—the Reviewer made the comment "Cellular process? homeostatic mechanism (provide your working definition of what homeostatic means) – see Chapter 2 for

several examples not reflected in this table where metal-metal interactions at the cellular level may occur."

RESPONSE: *Footnotes to Table 4 were changed to better describe the working definition of evidence for a homeostatic mechanism or process.*

For example:

> **+** = Evidence from studies of isolated membranes, cells, or tissues for potential coupling of at least one homeostatic mechanism/process.

COMMENT 67: Referring to the statement in the legend in Table 4—+++ = >5 studies indicating coupling at >2 homeostatic mechanisms/processes—the Reviewer made the comment "Why is the number of studies used as a surrogate for the strength of an interaction? This is hard to justify/."

RESPONSE: *Numbers of studies were removed from the revised weight-of-evidence scale; the revised scale is based on source of evidence (isolated membranes, cells, or tissues versus additional* in vivo *evidence) and number of homeostatic mechanisms identified. See example in the Response to Comment 66.*

COMMENT 68: Referring to the statement in Section 3.2—Hazard index approaches for exposure to mixtures containing manganese, iron, calcium, and magnesium and utilizing a hazard quotient for manganese should acknowledge the likely susceptibility of iron-deficient individuals to manganese toxicity…—the Reviewer commented "How is this incorporated in the actual calculation? Is this relevant for the contaminated water sources?"

RESPONSE: *ATSDR guidelines (2018) recommend that Hazard Index calculations be accompanied with a qualitative evaluation of the weight of evidence for interactions; a quantitative modification of the hazard index is not recommended.*

> ATSDR. 2018. Framework for assessing health impacts of multiple chemicals and other stressors (update). Atlanta, GA: Agency for Toxic Substances and Disease Registry. https://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf. December 22, 2020.

This recommendation is consistent with the first sentences of Section 3.2:

> Use of the recommended approaches should be accompanied with qualitative descriptions of uncertainties associated with the exposure assessment and hazard assessment. A key uncertainty associated with the use of the hazard index approach is the lack of data to assess whether or not dose addition provides an accurate prediction of toxic noncancer responses to mixtures with the selected metallic cations.

Section 3.1 was revised to clarify ATSDR guidelines on this issue:

> Following ATSDR (2004a, 2018) guidelines, a component-based hazard index approach is recommended assuming dose additivity for preliminary screening-level public health assessments. In the first tier of analysis (Tier 0), there is no grouping based on common toxicity targets or common adverse outcomes with common modes of action. In this approach, the ratios of exposure levels to health guidance values (hazard index) for each substance affecting a particular endpoint (hazard quotients) are summed to provide a measure of hazard for the whole mixture (see formulas for hazard quotients and hazard index below). Because it assumes dose addition, the hazard index is most appropriately applied to components that cause the same effect by the same mechanism of action (i.e., elicit a common adverse outcome via a common mode of

action). However, ATSDR (2004a, 2018) recommends (in the absence of hazard identification and dose-response data for a mixture of concern or a sufficiently similar mixture) using the hazard index approach for all components in a mixture regardless of toxicity target in an initial Tier 0 preliminary screening-level approach, followed by more complex tiers of analysis, involving progressive grouping of components based on common adverse outcomes (i.e., toxicity target) alone or common adverse outcomes via a common mode of action as indicated by available data on the components. The recommended dose-addition approach is a default approach for screening-level assessments that is supported by results from studies of cultured cells and laboratory animals exposed to various chemical mixtures indicating that deviations from dose addition (also known as concentration addition) have been relatively small from a risk assessment perspective (ATSDR 2018).

COMMENT 69: Referring to the discussion in Section 3.2 regarding TDD for neurological effects from barium, the Reviewer made the comment "As mentioned earlier modes of action and neurological targets are not the same. As mentioned earlier Fe and Mn do however share similar extrapyramidal pathology."

RESPONSE: *ATSDR recognizes that common modes of action and common toxicity targets are not the same. Throughout the document, sentences that may have been interpreted to mean that they were the same have been modified. For example:*

Section 2.2.6:

There is no clear evidence in humans or laboratory animals that repeated excess exposure to either of these metallic ions alone results in a common adverse outcome via a common mode of action or adverse effects in a common target organ or tissue (see Appendices A and G).

COMMENT 70: Referring to the statement in Section 3.2: Potential Influences on Sodium-Induced Hypertension—Evidence in human clinical trials, however, has been mixed, with some studies obtaining positive evidence of counteraction and others reporting no counteractive effect—the Reviewer made the comment "This literature has been incompletely described in the draft document."

RESPONSE: *In Section 3.2, the reader is referred to Sections 2.2.10 and 2.2.17 to support this statement about clinical trials of calcium or magnesium to counter hypertension. ATSDR disagrees with the Reviewer that the literature is incompletely described; the level of detail is appropriate for the intended scope of the interaction profile.*

COMMENT 71: Referring to the statement in Section 3.2—The available evidence for interactions between calcium and strontium is inadequate to conclude whether or not concomitant exposure to excess calcium and excess strontium will modify the potential for calcium to induce kidney stones…—the Reviewer commented "other minerals play an important dietary role in the development of calcium oxalate uroliths in animals."

RESPONSE: *See Responses to Comments 15 and 65, which outline revisions made in Appendix F that speak to this issue.*

COMMENT 72: Referring to the statement in Section 3.2—…magnesium as therapy against kidney stone recurrence are mixed: some trials suggested a protective effect and others reported no effect—the

Reviewer made the comment "see earlier comment about calcium oxalate uroliths, this statement needs to indicate species and again the studies cites are incompletely described in this review."

RESPONSE: *Text in Section 3.2 was revised to emphasize where the results under discussion are from human studies. See also Responses to Comments 15 and 65.*

> ***Potential Influences on Calcium-induced Kidney Stones and Possible Combined Kidney Toxicity.*** Magnesium has been shown to be an effective inhibitor of calcium oxalate stones *in vitro*, but results from clinical trials of dietary supplementation with magnesium as therapy against kidney stone recurrence in humans are mixed: some trials suggested a protective effect and others reported no effect (Section 2.2.8). The designated "limited evidence" for a protective effect of magnesium against calcium-induced kidney stones in Table 4 reflects the positive results obtained in these studies, but should be regarded as highly uncertain because the formation of calcium oxalate kidney stones is thought to be influenced by multiple factors (including age, sex, fluid intake, obesity, diabetes, citrate intake, extent of binding of oxalate in the intestine, urinary oxalate excretion, and potassium intake), and there is controversy on how to interpret the inconsistent evidence for increased calcium intake as a risk factor for kidney stone formation in humans (see Section 2.2.8 and Appendix B). For example, U.S. (NAS 2011) and European (EFSA 2012) agencies differ in their evaluation of the critical effect on which to base a UL for calcium. NAS (2011) based their value on a putative human "LOAEL" value, whereas EFSA (2012) determined that there was no reliable human "LOAEL" within the database for dietary calcium supplementation (see Appendix B). Emerging evidence indicates that coupling of homeostatic processes for calcium and magnesium exists at several sites (e.g., TRPV5-mediated calcium reabsorption in the renal distal tubule, CaSR, PTH secretion from the parathyroid), but current understanding of the complex and interacting homeostatic processes for calcium and magnesium is inadequate to explain how these potential coupling sites might work together under conditions of high oral intakes of both metallic cations, as is the case in the mixture of concern in waste water from UOG activities. Hazard index approaches utilizing a hazard quotient for calcium based on kidney stone formation should be accompanied by qualitative statements of the uncertainties associated with calcium's potential to induce kidney stones in humans and magnesium's potential to protect against kidney stone formation in humans.

COMMENT 73: Referring to the discussion in Appendix G.3, the Reviewer made the comment "This section does not provide a clear understanding of mechanisms at the cellular level that may be relevant for Sr interactions with Ca and other cations."

RESPONSE: *The Appendix is meant to give the reader access to a broad overview of toxicokinetics, toxicology, toxic modes of action, and available health guidance values. ATSDR is comfortable with the level of detail in this section. Some revisions to Section 2.2.11 (Calcium and Strontium) were made to better describe findings from research on possible interactions between calcium and strontium at the cellular level (see Response to Comment 9).*

COMMENT 74: The Reviewer made the following comment in Appendix G.5 "How are zebrafish data considered? e.g., some evidence that Sr is hepatotoxic see: Sci Total Environ. 2019 Jun 20;670:433-438."

RESPONSE: *The Appendix is meant to give the reader access to a broad overview of toxicokinetics, toxicology, toxic modes of action, and available health guidance values. The study referenced by the Reviewer would be pertinent to the assessments for health hazards from strontium; however, this study*

alone does not provide evidence to clearly identify strontium as a hepatotoxic agent in humans, the primary species of concern for all of ATSDR's assessments.

COMMENT 75: The Reviewer made the following comment in Appendix A.2 "Here and elsewhere – exposure doses are not provided so what is meant by "very high levels."

RESPONSE: *The referenced sentence in Appendix A.2 was modified by replacing, "very high levels" with "at undetermined doses" and adding "oral LD_{50} values in rodents range from about 130 to 279 mg/kg." The rest of this section includes dose levels associated with effects as reported in the source document (ATSDR 2007).*

> Potentially fatal hypokalemia (decreased potassium blood levels), resulting in ventricular tachycardia, hyper- and/or hypotension, muscle weakness, and paralysis, has been reported in individuals following accidental or intentional ingestion (at undetermined doses) of water-soluble barium compounds such as barium carbonate or chloride; oral LD_{50} values in rodents range from about 130 to 279 mg/kg (ATSDR 2007).

COMMENT 76: The Reviewer made the following comment in Appendix C.3 "Other studies have looked at Mn and Ba (and other cations) effects on neuronal nitric oxide synthase activity (Weaver et al., 2004; others)."

RESPONSE: *The Weaver et al. (2004) study was added to Section 2.2.4.*

> Also, barium, manganese, and nickel, like calcium, have been shown to activate purified nitric oxide synthase, a Ca^{+2}/calmodulin binding enzyme that mediates production of nitric oxide free radicals, which are thought to be involved with vasodilator tone, hypertension, and neuronal function (Weaver et al. 2004).

COMMENT 77: Referring to the statement in Appendix A.4—Several health guidelines have been established by various agencies to protect against adverse effects from inhalation exposure to barium…—the Reviewer made the comment "Not clear why inhalation data is included here and elsewhere especially for portal of entry effects."

RESPONSE: *Inhalation data or health guidance values were discussed in appendices to provide a brief overall description of toxicological properties.*

COMMENT 78: The Reviewer made the following comment in Appendix C.3 "As mentioned earlier ROS production is also a concern with certain other components – e.g., Mn."

RESPONSE: *Revisions were made other places in the document pointing out that iron and manganese share the property/mode of action of reactive oxygen species production.*

For example, Section 2.2.13:

> The nervous system may be a common toxicity target of excess manganese and excess iron, as The nervous system may be a common toxicity target of excess iron and excess manganese, as evidenced by observations of: (1) neurological dysfunction in human subjects with genetic disorders or neurodegenerative diseases such as Parkinson's and Alzheimer's disease and accumulation of iron, manganese, and other metals in brain tissue (Chen et al. 2019; Dusek et al. 2015a, 2015b; Huat et al. 2019); (2) neurological dysfunction and accumulation of manganese in

brains of human subjects with high occupational exposures to manganese (e.g., manganese miners, see ATSDR 2012); and (3) common proposed toxic actions related to generation of reactive oxygen species (see Appendices C and E).

COMMENT 79: Referring to the statement in Appendix B.3—Other proposed mechanisms include free radical formation, oxidative stress, alterations in mitochondrial energy metabolism, disruption of cellular iron and calcium homeostasis…—the Reviewer made the comment "But Ca2+ seemingly ignored in the binary mixture discussion."

RESPONSE: *No changes necessary here. Revisions were made to a number of Chapter 2 subsections describing interactions of the other subject metallic cations with calcium channels and calcium regulatory proteins.*

For example, Section 2.2.11:

Many studies have examined the ability of strontium (and other divalent cations) to permeate through and modulate activity of isolated calcium transport systems (e.g., Bouron et al. 2015; Nelson 1986; Potreau et al. 1987; Tsien et al. 1987). In many systems, strontium is permeable (and could potentially compete with calcium) through calcium channels and can compete with calcium at allosteric binding sites. However, due to the complexity of calcium homeostasis at cellular, tissue, and whole-organism levels of organization, the significance of these types of findings to the possible joint action of calcium and strontium on toxicity targets after combined oral exposure of animals or humans is unclear.

Comments provided by Peer Reviewer #3:

General Comment

COMMENT 1: As requested, the analysis of the report mostly focused on magnesium.

RESPONSE: *No response necessary.*

ATSDR Charge Questions and Responses

Chapter 1

QUESTIONS:

1) Do you agree with those effects known to occur in humans as reported in the text? If not, please explain why and provide a copy of additional references you would cite and indicate where (in the text) these references should be included.
2) Are the effects only observed in animals likely to be of concern to humans? Why or why not? If you do not agree, please explain.
3) Have exposure conditions been adequately described? If you disagree, please explain.

COMMENT 2: The answer is "Yes" to all three questions.

RESPONSE*: No response necessary.*

COMMENT 3: The reviewer has three specific comments. The focus has been put on chronic toxicity and no acute toxicity is considered; however, based on the values of cations provided in Tables I, acute toxicity should also be considered: actually acute ingestion of water containing large amounts of Na and Ca may induce substantial and rapid changes in body Na and Ca content, potentially hazardous.

RESPONSE: *ATSDR appreciates the comment that acute effects from the metals at the concentrations detected in waste waters from UOG extraction activities would be of concern, but recognizes that release of such waters into groundwater and subsequent transport to drinking water wells would lead to some dilution. As such, ATSDR believes that the chronic exposure to contaminated groundwater and potential drinking water wells remains the exposure scenario of most concern.*

COMMENT 4: Potassium is not considered: is the reason that potassium does not contaminate water in UOG plants? Or the potassium contents have not been measured?

RESPONSE: *Potassium is not considered in this profile, because it has not been listed in published reports of chemical compositions of UOG extraction waste water samples listed in Appendix I.*

COMMENT 5: Many cations are eliminated from the body by the kidney into the urine, which maintains homeostasis. Patients with chronic kidney disease and reduced GFR have a progressive impairment to eliminate the adequate amount of solutes into urine. Those people should be at higher risk of toxic effects of contaminated water. This should be mentioned in the introduction.

RESPONSE: *This point was added to recommendations in Section 3.1.*

Because the kidney plays a key role in whole-body homeostasis for most of these metallic cations, people with kidney functional abnormalities or disease are expected to be especially susceptible to their toxicities. Use of the recommended hazard index approaches in public health assessments should be accompanied with qualitative statements that the assessments may not be protective for such individuals.

Chapter 2

QUESTION:

1) Do the health effect conclusions made in Chapter 2 adequately reflect the findings in the published literature? If not, please suggest appropriate changes.
2) Were adequately designed human studies identified in the text, if available (i.e., good exposure data, sufficiently long period of exposure to account for observed health effects, adequate control for confounding factors)? Were the major study limitations sufficiently described in the text without going into lengthy discussions? If study limitations were not adequately addressed, please suggest appropriate changes.
3) Were adequately designed animal studies identified in the text (i.e., adequate number of animals, good animal care, accounting for competing causes of death, sufficient number of dose groups, and sufficient magnitude of dose levels)? If not, does the inadequate design negate the utility of the study? Please explain.
4) Were the animal species appropriate for the most significant toxicological endpoint of the study? If not, which animal species would be more appropriate and why?
5) Has adequate attention been paid to dose-response relationships for both human and animal data? Please explain.
6) Are you aware of any studies that are not included in the profile that may be important in evaluating the toxicity of the substance? Please provide a copy of each study and indicate where in the text each study should be included.
7) Are you aware of any studies that are not included in the profile that may be relevant to deriving MRLs for any of the substance isomers? Please provide a copy if this is a new reference.
8) Are the conclusions appropriate given the overall database? If not, please discuss your own conclusions based on the data provided and other data provided to you but not presented in the text.
9) Does the binary approach seem reasonable?

COMMENT 6: The purpose of the section was to describe the potential effect of the considered cations when they are simultaneously present in waste water. The approach used in this chapter is a second best option, as no study is available that considered combination of all 7 cations. As acknowledged by the authors, the best they could find was studies where2 out of 7 cations were simultaneously changed.

Considering Mg, the signs and symptoms of Mg overload are usually not apparent until Mg concentration is in excess of 2 mmol/L (Mordes JP, Wacker WEC, Pharmacol rev 1978; 29: 273-300), a value which is difficult to reach by oral intake only and which is usually the consequence of high parenteral infusion. However, as mentioned by the authors, cases of hypermagnesemia have been occasionally described. A recent study described the effect of chronic treatment with MgO, as laxative (mean dosage 1040 ± 430 mg) in 193 adult patients ((PMID 31379418); 5.2 % of the patients had mild hypermagnesemia (plasma Mg >3 mg/dL) but none had plasma Mg > 4.8 mg/dL. A recent report describes a nearly fatal hypermagnesemia (14.3 mg/dL) after the ingestion of 34 g Mg citrate over 2 days for preoperative colon preparation (PMID 26181446). MgO can be used in preparations for colon cleansing before colonoscopy. In healthy volunteers, after having taken 2 sachets of Pico-Salax, providing 7 g MgO over 5 hours, plasma

Mg concentrations increased but were not higher than 1.2 mmol/L over the 24 hours following Mg administration (PMID 22803016). Several cases of hypermagnesemia, sometimes fatal, after ingestion of massive dose of Mg salts have been reported in the literature. However, in all those studies, the effects of other cations accompanying magnesium are not considered.

RESPONSE: *No response necessary.*

Specific Comment on Chapter 2

COMMENT 7: Binary mixtures of components. When considering the impact of a mixture of components in the context of water contamination, the studies in which one of the component is deficient are probably not relevant (actually, it cannot be derived from the fact that Mg deficiency favors a process that Mg excess will inhibit it); based on data in Table I-1, the concentration of Mg in effluent water can be as high as ~100 mM, a concentration very rarely considered in experimental studies.

Response: *ATSDR agrees with the comment that 100 mM Mg concentrations have been rarely used in experimental studies, but thinks that some dilution of waste waters will occur upon release into groundwater and transport into drinking water wells as noted in the Response to Comment 3. In Chapter 2, ATSDR discussed studies examining the influence of deficiency of one metal on the disposition or toxicity of another metal to illustrate interactions and couplings of homeostatic mechanisms of the metals. No changes were made in response to the comment above.*

COMMENT 8: Regarding the effects of Na excess, a common misunderstanding is to consider that an excess in Na intake would directly affect Na-dependent transport processes in plasma membranes (e.g. Na/Mg exchange). Actually, the main effect of excessive Na intake is to increase the Na stored in the body, mainly in the extracellular fluid volume, but it is very rare that an excess of Na intake affects the extracellular fluid Na concentration and, therefore, the driving force for Na-dependent transport processes. An exception is the condition of highly hyperosmotic intake, not compensated for by appropriate intake of fresh water. However, a high Na intake has many neuro-hormonal effects (e.g. inhibition of renin release, inhibition of aldosterone release, stimulation of endolthelin-1 release, etc…) that can secondarily affect the function of several membrane transporters involved in Na homeostasis but also in the homeostasis of other ions. A good example of this is the increase in urinary calcium excretion that accompanies consistenly the increase in Na intake, because the increase in extracellular fluid volume caused by high Na intake decreases renal tubular calcium reabsorption.

Overall, as repeatedly mentioned by the authors of the report, no study is available that adequately assess the effects of combined high intakes of the cations considered in the report and of magnesium, especially at intake values as high as those reported in Table I-1 (up to 117 g Na, 41 g Ca, 13.8 g Ba, 8.5 g Sr). However, it is likely that such high levels of cations, when simultaneously present, have significant, but difficult to predict, impact on body ion homeostasis.

Additional, targeted, studies are needed to assess the potential effects of combined high concentrations of cations on the ability to maintain homeostasis; in the presence of amounts as high as those reported in table I-1, it is likely that the homeostatic systems will be overwhelmed and that significant consequences will be apparent.

RESPONSE: *As discussed above, ATSDR expects people to be exposed to contaminated water at concentrations below those in the tables in Appendix I; these are concentrations in the waste waters before they are released into the environment (i.e., groundwater and potential transport to drinking water*

wells). However, discussion has been added to Section 2.1 and Chapter 3 to discuss the limitations of the available interaction data on binary mixtures to assess combined simultaneous exposure to all seven metallic cations due to the overlapping nature of homeostatic mechanisms for metals and the need for targeted studies involving three or more of the subject metals.

Section 2.1:

No studies were located examining toxicokinetic or toxicological endpoints in humans or laboratory animals exposed to mixtures containing more than two of the subject metallic cations and comparing the responses to responses from sole exposures to the individual cations. As a result, available interaction data for binary mixtures of the components were evaluated in Section 2.2.

The limitation of this binary approach is recognized due to evidence that overlap in homeostatic mechanisms can occur for more than two metals.

Section 3.2:

For this document, data on potential interactions between pairs of the selected metallic cations were identified and evaluated to assess evidence that could qualitatively modify public health assessments (using component-based hazard index approaches) for mixtures with the metallic cations of concern. A summary of this analysis (presented in Chapter 2) follows. This binary approach is acknowledged to be a practical approach with inherent uncertainty due to evidence that coupling of metallic cation homeostatic mechanisms is complex and can overlap for two or more metals.

Section 3.3:

The recommended component-based hazard index approach and binary evaluation of interaction data is acknowledged to be a practical approach with inherent uncertainty due to evidence that coupling of metallic cation homeostatic mechanisms is complex and can overlap for two or more metals.

Chapter 3

QUESTION:

1) Is there adequate discussion of the complex interactive effects and other substances? Does the discussion concentrate on those effects that might occur at hazardous waste sites? Please explain and provide any additional references.
2) If interactive effects with other substances are known, does the text discuss the mechanisms of these interactions? Please explain and provide any additional references.
3) Is this approach reasonable?

COMMENT 9: This reviewer is not familiar with the field of toxicology, but the rationale basis for the use of an additive approach is not quite clear. For example, in the presence of barium-induced hypokalemia, the occurrence of acute hypercalcemia due to ingestion of high calcium-containing water is likely to increase non linearly the risk of cardiac arrhythmia: regarding the effect of electrolyte disorders, the relation between the severity of the disorder and the risk is usually non linear.

The risk related to high Na intake is mainly hypertension (if it is chronic) or acute hypernatremia (if it is acute); this reviewer is unsure that it makes sense to add the risk related to hypertension and that related to barium-induced hypokalemia and/or calcium-induced hypercalcemia, as the later will mainly increase the risk of cardiac arrhythmia whereas the former will increase the risk of vascular (including coronary) event.

RESPONSE: *ATSDR believes that the intended users of this profile, toxicologists and public health assessors, will understand the current text describing the uncertainties and limitations associated with the recommended component-based hazard index approach (which assumes dose addition) and the rationale for choosing this approach in the absence of toxicological studies and dose-response data for mixtures containing the seven metallic cations. We agree with the Reviewer's apparent concern about combining risk across different toxicity targets and effects with different modes of action, but believe that targeted readers of the report will understand the rationale for using a component-based approach in the absence of "whole-mixture" data as articulated in ATSDR 2004a, 2018.*

ATSDR. 2004a. Guidance manual for the assessment of joint toxic action of chemical mixtures. Agency for Toxic Substances Disease Registry. http://www.atsdr.cdc.gov/interactionprofiles/IP-ga/ipga.pdf. September 8, 2015.

ATSDR. 2018. Framework for assessing health impacts of multiple chemicals and other stressors (update). Atlanta, GA: Agency for Toxic Substances and Disease Registry. https://www.atsdr.cdc.gov/interactionprofiles/ip-ga/ipga.pdf. December 22, 2020.

Chapter 4

QUESTION:
1) Are these conclusions reasonable? Please explain and provide any additional comments.
2) Is information provided based upon current understanding of the literature?

COMMENT 10: As mentioned above, as mentioned by the authors of the report, no study is available that adequately assess the effects of combined high intakes of the cations considered in the report and of magnesium, especially at intake values as high as those reported in Table I-1 (up to 117 g Na, 41 g Ca, 13.8 g Ba, 8.5 g Sr). However, it is likely that such high levels of cations, when simultaneously present, have significant, but difficult to predict, impact on body ion homeostasis.

Additional, targeted, studies are needed to assess the potential effects of combined high concentrations of cations on the ability to maintain homeostasis; in the presence of amounts as high as those reported in table I-1, it is likely that the homeostatic systems will be overwhelmed and that significant consequences will be apparent.

RESPONSE: *ATSDR agrees that additional targeted studies are needed to better assess potential effects of combined high concentrations of the subject cation on the ability to maintain homeostasis and to produce toxic effects, and thinks that this need is adequately described in Section 3.3, Data Needs.*

Chapter 5

QUESTION:
1) Are you aware of any additional references that should be included? Please provide citations.
2) Please note the suggested location in text that new articles should be cited.

COMMENT 11: The authors could add to recent reviews regarding Mg homeostasis

Houillier P. Mechanisms and regulation of renal magnesium transport. Annu Rev Physiol. 2014;76:411-30.

de Baaij JH, Hoenderop JG, Bindels RJ. Magnesium in man: implications for health and disease. Physiol Rev. 2015 Jan;95(1):1-46.

RESPONSE: *The suggested articles were retrieved and examined. Information in these and other recent review articles were used to address the Reviewer's comments on Appendix G (see Comment 13 below).*

Appendix I

COMMENT 12: Appendix I provides 3 tables summarizing available data regarding Mg content in waste water from UOG; Mg concentration ranges from 17 to 2550 (average 632) mg/L (Table I-1 Pennsylvania Marcellus Shale), and from 77 to 263 mg/L (Table I-2). The discrepancy between data from Table I-1 and TableI-2 is unclear, as Pennsylvania Marcellus Shale is one of the activities considered in Table I-2. Table I-3 provides evidence that the high concentration of Mg in waste fluid is actually a contamination, as it is much lower in injected fluid (3.7 mg/L). Note that the values of Na content are in the range of what can be measured in sea water.

RESPONSE: *The data in Appendix I tables are presented to give the reader an idea of the variability in reported compositional data for metallic cations in waste water from UOG extraction activities in different regions of the United States. No changes were made in response to these comments; ATSDR thinks that the tables describe the variability adequately.*

Appendix D (previously Appendix G)

COMMENT 13: Appendix G summarizes the background information for magnesium.
In a first part, it describes the known roles magnesium in mammals, putting the emphasis on the role of Mg-ATP, Mg interaction with enzyme activities and Mg transport across plasma membranes. The normal ranges of Mg in extracellular fluid and cytosol should be added (e.g. ~1 mM in ECF, ~20 mM in cytosol, of which 5% is in the free form). In the toxicokinetics part, it is unclear what the authors means by"… is absorbed into intestinal cells by passive membrane transport system driven by electrocheminal gradients and, to a lesser degree, by active, saturable systems": actually, transcellular Mg absorption is a 2-step process, with a passive apical transport (along the electrochemical gradient) and an active basolateral extrusion, against the electrochemical gradient; to maintain cell content in Mg, both processes need to transport Mg at the same rate. In addition, a passive paracellular absorption process does exist, likely more in the small intestine than in the colon. Page 116, lines 12-15: the contribution of the proximal tubule to renal tubular Mg reabsorption should be mentioned, although proximal Mg absorption is probably not under hormonal regulation.

RESPONSE: *Articles recommended by the Reviewer above (De Baaij et al. 2015 and Houillier et al. 2014) were retrieved and examined. Based on information in these and other more recent articles, text in the toxicokinetic section of Appendix D was revised to better reflect current understanding of magnesium homeostasis at cellular and organismal levels of organization.*

> Saturable transport across intestinal cells has been proposed to occur primarily in the colon and involve transient receptor potential melastatin subtype 6 and 7 (TRPM6, TRPM7) channels for luminal uptake, CNNM4 channels for basolateral extrusion and driving forces from sodium gradients (de Baaij et al. 2015; Houillier 2014). The paracellular pathway has been proposed to proceed through tight junctions between cells in the small and large intestine whose permeability is influenced by multimolecular complexes containing claudins (de Baaij et al. 2015; Houillier 2014). Which claudins are involved in magnesium permeability and how they are regulated is under investigation (de Baaij et al. 2015; Houillier 2014).

Numerous transport proteins that are thought to be involved in magnesium homeostasis include transient receptor potential M6 and M7 ion channel kinases (TRPM6, TPPM7), Na+/K+ ATPases, Solute Carrier family 41 member A1 (SLC41A1), and Cystathionine-β synthase (CBS)-pair domain divalent metal cation transport mediators (CNNMs: e.g., CNNM2) (Arjona and de Baaij 2018; Arjona et al. 2019; de Baaij et al. 2015; Funato et al. 2018; Garcia-Castano et al. 2020; Flatman 1991; Kolisek et al. 2019; Mittermeier et al. 2019; Romani 2011; Schlingmann et al. 2007, 2018; Watanabe et al. 2005; Workinger et al. 2018).

COMMENT 14: Therapeutic use of Mg should be expanded to conditions (usually rare monogenic diseases) with hypomagnesemia due to impaired intestinal absorption of Mg (e.g. TRPM6 loss of function mutations) and/or massive renal leak of Mg (e.g. Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis caused by CLDN16 loss of function mutations).

RESPONSE: *A statement was added to the paragraph in Appendix D, Section D.2 on therapeutic uses of supplementary magnesium in treating patients with genetic disorders associated with hypomagnesemia and mutations in genes for proteins involved in magnesium homeostasis, as discussed in the article by De Baaij et al. (2015) and suggested by the Reviewer.*

Therapeutic uses of magnesium compounds include: (1) intravenously administered magnesium sulfate to prevent convulsions in women with severe preeclampsia, the mechanism of which is not understood (Belfort et al. 2003; Greene 2003; The Magpie Trial Collaborative Group 2002); (2) 24-hour intravenous magnesium sulfate to patients with suspected acute myocardial infarction, presumably by inhibiting calcium influx into ischemic myocardial cells, and thereby reducing injury, arrhythmias, and post-event mortality (Antman 2002; ISIS 1995; Woods 1991; Woods and Fletcher 1994); (3) oral administration of magnesium sulfate or other easily dissociable magnesium compounds (e.g., magnesium chloride, magnesium oxide) to empty the intestine from an osmotic effect (EFSA 2006, 2015b; NAS 1997; Swaminathan 2003); and (4) oral or intravenous magnesium supplementation has been used to treat hypomagnesemia in patients with mostly rare mutations in genes encoding proteins involved in magnesium homeostasis (see de Baaij et al. 2015 for review).

COMMENT 15: Starting on page 119, line 1, the paragraph on the effects of low Mg intake/Mg deficiency is probably not relevant in this review. However, the authors should mention that the Mg concentration found in waste water from UOG is, in some cases (e.g. Table I-1), above the NOAEL.

RESPONSE: *ATSDR thinks that the paragraph in Appendix D, Section D.2 is very important to describe the rationale and approach used to derive the magnesium UL, and made no changes in response to this comment. The average magnesium concentrations in Appendix I tables (in units of mg Mg/L) are not directly comparable to the NOAELs (in units of mg Mg/day), but assuming 2 L water consumed /day, average intakes of 1,246 mg/day can be calculated. Such calculated intakes are above the NOAEL values discussed in the paragraphs, but the comparison is not pertinent to the meaning of this paragraph and surrounding paragraphs describing the rationale and approach to derive the UL.*

Appendix D, Section D.2:

In a more recent review, EFSA (2006) identified 20 reports of examinations of mild diarrhea in subjects ingesting daily oral magnesium supplements with doses ranging from 180 to 1,095 mg Mg/day for durations ranging from 1 to 72 weeks. All of these studies only reported magnesium doses in the supplements; they did not report magnesium intakes from food or beverages. Four studies with daily doses ranging from 180 to 250 mg Mg/day reported no mild diarrhea in 130

subjects (Classen et al. 1986, as cited in EFSA 2006), 112 subjects (Schimatschek et al. 1997, as cited in EFSA 2006), 181 subjects (Schimatschek et al. 2001, as cited in EFSA 2006), or 31 subjects (Stendig-Lindberg et al. 1993). The first three studies administered magnesium aspartate hydrochloride daily for 3 weeks, and the last study administered magnesium hydroxide daily for 72 weeks. At least one case of mild diarrhea occurred in four of six studies that administered daily doses of 360–365 mg Mg/day for 4 to 26 weeks to groups with 17–278 subjects (Cappuccio et al. 1985; Fehlinger et al. 1988; Gullestad et al. 1992; Plum-Wirell et al. 1994; Spätling and Spätling 1988; Rasmussen et al. 1989, as cited in EFSA 2006). Daily doses ranged from 384 to 1,095 mg Mg/day in the remaining 10 studies, of which 8 studies reported at least one subject with mild diarrhea (Bashir et al. 1993; Daly and Harris 1990; Marken et al. 1989; Muehlbauer et al. 1991, as cited in EFSA 2006; Nadler et al. 1992; Ricci et al. 1991; Ruddel et al. 1990; Spätling et al. 1998; Spencer et al. 1994; Widman et al. 1993). Based on its review of results from these 20 studies of adult men and women (some of which included pregnant and lactating women), EFSA (2006) concluded that 250 mg Mg/day was a NOAEL for laxative effects from nonfood readily dissociable magnesium salts or magnesium oxide. This NOAEL was used as the basis for a recommended UL for magnesium that does not include magnesium present in foods or beverages (EFSA 2006).

Comments provided by Peer Reviewer #4:

ATSDR Charge Questions and Responses

Chapter 1

QUESTION: Do you agree with those effects known to occur in humans as reported in the text? If not, please explain why and provide a copy of additional references you would cite and indicate where (in the text) these references should be included

COMMENT 1: Yes.

RESPONSE: *No response needed.*

QUESTION: Are the effects only observed in animals likely to be of concern to humans? Why or why not? If you do not agree, please explain.

COMMENT 2: While it is difficult to conclude with certainty, clearly the results from animal studies should be of concern to human health.

RESPONSE: *No response needed.*

QUESTION: Have exposure conditions been adequately described? If you disagree, please explain.

COMMENT 3: *Yes*

RESPONSE: *No response needed.*

Chapter 2

QUESTION: Do the health effect conclusions made in Chapter 2 adequately reflect the findings in the published literature? If not, please suggest appropriate changes.

COMMENT 4: *Yes.*

RESPONSE: *No response needed.*

QUESTION: Were adequately designed human studies identified in the text, if available (i.e., good exposure data, sufficiently long period of exposure to account for observed health effects, adequate control for confounding factors)? Were the major study limitations sufficiently described in the text without going into lengthy discussions? If study limitations were not adequately addressed, please suggest appropriate changes.

COMMENT 5: Yes.

RESPONSE: *No response needed.*

QUESTION: Were adequately designed animal studies identified in the text (i.e., adequate number of animals, good animal care, accounting for competing causes of death, sufficient number of dose groups, and sufficient magnitude of dose levels)? If not, does the inadequate design negate the utility of the study? Please explain.

COMMENT 6: Yes.

RESPONSE: *No response needed.*

QUESTION: Were the animal species appropriate for the most significant toxicological endpoint of the study? If not, which animal species would be more appropriate and why?

COMMENT 7: Yes.

RESPONSE: *No response needed.*

QUESTION: Has adequate attention been paid to dose-response relationships for both human and animal data? Please explain.

COMMENT 8: Yes.

RESPONSE: *No response needed.*

QUESTION: Are you aware of any studies that are not included in the profile that may be important in evaluating the toxicity of the substance? Please provide a copy of each study and indicate where in the text each study should be included.

COMMENT 9: I find the discussions on interactions between 2 metals to be adequate, but in real-life scenarios, exposures occur to multiple metal mixtures. The idea that the relationships between the metals can be separated to 2 at a time, does not reflect the reality of exposures. For example:

> Toxicol Sci. 2006 Aug;92(2):516-25. Epub 2006 May 16.
> A manganese-enhanced diet alters brain metals and transporters in the developing rat.
> Garcia SJ[1], Gellein K, Syversen T, Aschner M.
> **Author information**
> **Abstract**
> Manganese (Mn) neurotoxicity in adults can result in psychological and neurological disturbances similar to Parkinson's disease, including extrapyramidal motor system defects and altered behaviors. However, virtually nothing is known regarding excess Mn accumulation during central nervous system development. Developing rats were exposed to a diet high in Mn via maternal milk during lactation (PN4-21). The high Mn diet resulted in changes in hematological parameters similar to those seen with iron (Fe) deficiency in dams (decreased plasma Fe; increased plasma transferrin [Tf]) and pups (decreased hemoglobin [Hb] and plasma Fe; increased plasma Tf and total iron binding capacity). Mn-exposed pups showed an increase in brain Mn, chromium, and zinc concurrent with a decrease in brain Fe. In conjunction with the

altered transport and distribution of essential metals within the brain, there was enhanced protein expression of the divalent metal transporter-1 (DMT-1) and transferrin receptor (TfR) overall in the brain; there was a general increase in each region analyzed (cerebellum, cortex, hippocampus, midbrain, and striatum). Neurochemical changes were observed as an increase in gamma-aminobutyric acid (GABA) and the ratio of GABA to glutamate, indicating enhanced inhibitory transmission in the brain. The results of this study demonstrate that developing rats undergo alterations in the transport and distribution of essential metals translating to neurochemical perturbations after maternal exposure to a diet supplemented with excess levels of Mn.

This paper shows that changes in Mn or Fe do not only affect each others concetrations in the brain. As noted Mn-exposed pups showed an increase in brain Mn, chromium, and zinc concurrent with a decrease in brain Fe. Therefore, the discussion should at least acknowledge that ata this point the literature does not permit analysis beyond the relationships between 2 metals, but that likely, in real-life-scenarios, changes in a single metal levels are going to have multiple effects on many other metals. The 1:1 relationship is rather narrow in scope.

Another paper for consideration:

NMR Biomed. 2009 May;22(4):391-404. doi: 10.1002/nbm.1348.
A model for the analysis of competitive relaxation effects of manganese and iron in vivo.
Zhang N[1], Fitsanakis VA, Erikson KM, Aschner M, Avison MJ, Gore JC.
Author information
Abstract
Manganese (Mn) and iron (Fe) are both paramagnetic species that can affect magnetic resonance relaxation rates. They also share common transport systems in vivo and thus in experimental models of metal exposure their effects on relaxation rates may interact in a complex fashion. Here we present a novel model to interpret the combined effects of Mn and Fe on MRI relaxation rates. To achieve varying levels of both metals, adult rats were separated into four groups; a control group and three groups treated with weekly intravenous injections of 3 mg Mn/kg body for 14 weeks. The three treated groups were fed either a normal diet, Fe deficient or Fe enriched diet. All rats were scanned using MRI at the 14th week to measure regional water relaxation rates. Rat brains were removed at the end of the study (14th week) and dissected into regions for measurement of Mn and Fe by atomic absorption spectroscopy. For the normal diet groups, R(1) was strongly correlated with tissue Mn concentrations. However, the slopes of the linear regression fits varied significantly among different brain regions, and a simple linear model failed to explain the changes in relaxation rate when both Mn and Fe contents changed. We propose a competition model, which is based on the ability of Mn and Fe to compete in vivo for common binding sites. The combined effect of Mn and Fe on the relaxation rates is complicated and additional studies will be necessary to explain how MRI signals are affected when the levels of both metals are varied.

We have previously developed a model to interpret the combined effects of Mn and Fe on MRI relaxation rates. The slopes of the linear regression fits varied significantly among different brain regions, and a simple linear model failed to explain the changes in relaxation rate when both Mn and Fe contents changed. While this is corroborating the discussion in the document that Fe and Mn compete with each other for brain deposition, the findings also establish that the combined effect of Mn and Fe on the relaxation rates is complicated and that additional studies will be necessary to explain how MRI signals are affected when the levels of both metals are varied. The document needs to consider those changes and how potentially changes in brain Mn deposition under conditions of Fe deficiency may be more prevalent is some regions, adding a layer of complexity to assessing health risk.

Finally, in terms of health effects it is starting to become evident that the sum toxicity of 3 metals, may be greater than just one or two. This should also be noted.

> Biol Trace Elem Res. 2014 Jun;158(3):384-91. doi: 10.1007/s12011-014-9954-2. Epub 2014 Apr 9.
> Arsenic and manganese alter lead deposition in the rat.
> Andrade V[1], Mateus ML, Santos D, Aschner M, Batoreu MC, Marreilha dos Santos AP.
> **Author information**
> **Abstract**
> Lead (Pb) continues to be a major toxic metal in the environment. Pb exposure frequently occurs in the presence of other metals, such as arsenic (As) and manganese (Mn). Continued exposure to low levels of these metals may lead to long-term toxic effects due to their accumulation in several organs. Despite the recognition that metals in a mixture may alter each other's toxicity by affecting deposition, there is dearth of information on their interactions in vivo. In this work, we investigated the effect of As and Mn on Pb tissue deposition, focusing on the kidney, brain, and liver. Wistar rats were treated with eight doses of each single metal, Pb (5 mg/Kg bw), As (60 mg/L), and Mn 10 mg/Kg bw), or the same doses in a triple metal mixture. The kidney, brain, liver, blood, and urine Pb, As, and Mn concentrations were determined by graphite furnace atomic absorption spectrophotometry. The Pb kidney, brain, and liver concentrations in the metal-mixture-treated group were significantly increased compared to the Pb-alone-treated group, being more pronounced in the kidney (5.4-fold), brain (2.5-fold), and liver (1.6-fold). Urinary excretion of Pb in the metal-mixture-treated rats significantly increased compared with the Pb-treated group, although blood Pb concentrations were analogous to the Pb-treated group. Co-treatment with As, Mn, and Pb alters Pb deposition compared to Pb alone treatment, increasing Pb accumulation predominantly in the kidney and brain. Blood Pb levels, unlike urine, do not reflect the increased Pb deposition in the kidney and brain. Taken together, the results suggest that the nephro- and neurotoxicity of "real-life" Pb exposure scenarios should be considered within the context of metal mixture exposures.

> Biol Trace Elem Res. 2015 Jul;166(1):13-23. doi: 10.1007/s12011-015-0267-x. Epub 2015 Feb 20.
> Lead, Arsenic, and Manganese Metal Mixture Exposures: Focus on Biomarkers of Effect.
> Andrade VM[1], Mateus ML, Batoréu MC, Aschner M, Marreilha dos Santos AP.
> **Author information**
> **Abstract**
> The increasing exposure of human populations to excessive levels of metals continues to represent a matter of public health concern. Several biomarkers have been studied and proposed for the detection of adverse health effects induced by lead (Pb), arsenic (As), and manganese (Mn); however, these studies have relied on exposures to each single metal, which fails to replicate real-life exposure scenarios. These three metals are commonly detected in different environmental, occupational, and food contexts and they share common neurotoxic effects, which are progressive and once clinically apparent may be irreversible. Thus, chronic exposure to low levels of a mixture of these metals may represent an additive risk of toxicity. Building upon their shared mechanisms of toxicity, such as oxidative stress, interference with neurotransmitters, and effects on the hematopoietic system, we address putative biomarkers, which may assist in assessing the onset of neurological diseases associated with exposure to this metal mixture.

RESPONSE: *ATSDR agrees that the binary approach has limitations due to overlapping homeostatic mechanisms for metals that should be mentioned. Discussion was added to Sections 2.1, 2.2, 3.2, and 3.3*

and Chapter 4 to illustrate the limitations of the binary approach. ATSDR has reviewed and cited the reports suggested by the Reviewer (Andrade et al. 2014; Garcia et al. 2007).

For example (Section 2.1):

> The limitation of this binary approach is recognized due to evidence that overlap in homeostatic mechanisms can occur for more than two metals. For example, in rat pups exposed to high dietary manganese via maternal milk on postnatal days (PNDs) 4–21, brain concentrations of chromium, manganese, and zinc were increased and brain iron concentration was decreased (Garcia et al. 2007; see Section 2.2.13 for more details). Another example comes from a study of rats given eight daily oral doses of single metallic cations (arsenic, lead, or manganese) and a mixture of all three metallic cations, which showed that kidney, brain, and liver concentrations of lead in the metal-mixture-treated group were significantly increased by 5.4-, 2.5-, and 1.6-fold, respectively, compared to the lead-alone-treated group (Andrade et al. 2014).

QUESTION: Are you aware of any studies that are not included in the profile that may be relevant to deriving MRLs for any of the substance isomers? Please provide a copy if this is a new reference.

COMMENT 10: No.

RESPONSE: *No response needed.*

QUESTION: Are the conclusions appropriate given the overall database? If not, please discuss your own conclusions based on the data provided and other data provided to you but not presented in the text.

COMMENT 11: Per above, the discussion should be broadened with the acknowledgement that the changes seen likely go far beyond the 1:1 relationships developed in the document.

RESPONSE: *See response to comment 9 above.*

QUESTION: Does the binary approach seem reasonable?

COMMENT 12: Yes. But the limitation needs to be acknowledged and await future studies.

RESPONSE: *See response to comment 9 above.*

Chapter 3

QUESTION: Is there adequate discussion of the complex interactive effects and other substances? Does the discussion concentrate on those effects that might occur at hazardous waste sites? Please explain and provide any additional references.

COMMENT 13: Yes.

RESPONSE: *No response needed.*

QUESTION: If interactive effects with other substances are known, does the text discuss the mechanisms of these interactions? Please explain and provide any additional references.

COMMENT 14: The discussion is adequate.

RESPONSE: *No response needed.*

QUESTION: Is this approach reasonable?

COMMENT 15: The approach is reasonable, but it may underestimate the toxic effects, because as noted above, metal homeostasis goes beyond the 1:1 approach that is inherent to this document. The approach is reasonable based on the current state of knowledge, but the pitfalls should be noted.

RESPONSE: *See response to comment 9 above.*

Chapter 4

QUESTION: Are these conclusions reasonable? Please explain and provide any additional comments.

COMMENT 16: Yes. But I would hope some of the points I made above can be added to the conclusions.

RESPONSE: *Text in Chapter 4 was amended to point out the limitations of the binary approach.*

The hazard index approach is appropriate when the hazard quotients of at least two of the components are ≥0.1 (ATSDR 2004a, 2018). The hazard index approach includes making qualitative statements about associated uncertainties including: (1) evidence about possible interactions among the components of the mixtures that may deviate from the hazard predicted by dose addition and (2) the likelihood that estimates of hazard using this approach may not be protective for people with compromised kidney function, important for the normal, whole-body homeostasis of the five metals under consideration which are essential elements. The recommended hazard index approaches with their accompanied qualitative statements of uncertainty are default approaches supported by results from studies of cultured cells and laboratory animals exposed to various chemical mixtures indicating that deviations from dose addition have been small in these cases (ATSDR 2018). When the screening criteria are exceeded (hazard index >1), further evaluation is needed, using biomedical judgment and community-specific health outcome data and taking into account community health concerns (ATSDR 2004a, 2018).

Data on potential interactions between pairs of the selected metallic cations were identified and evaluated in Chapters 2 and 3 to assess evidence that could qualitatively modify public health assessments using component-based hazard index approaches for mixtures with the metallic cations of concern. The binary approach is acknowledged to be a practical approach with inherent uncertainty due to evidence that coupling of metallic cation homeostatic mechanisms is complex and can overlap for two or more metals.

Evidence for coupling of homeostatic mechanisms at the cellular level was available for all 21 pairs of the selected metallic cations; evidence for coupling at the whole-body level of organization was available for 12 pairs. The physiological and toxicological relevance of the findings from *in vitro* studies is not always clear, especially with regard to making reliable

predictions of how repeated combined oral exposure to any pair of metallic cations may influence their individual toxicity or indicate how they may jointly act on a shared toxicity target. For binary weights of evidence (BINWOEs) making a directional prediction, a small fraction (5%) was explained by *ex vivo* interactions (Pryzbyla et al. 2021). In contrast to the relative wealth of evidence for homeostatic coupling among the seven metallic cations, limited evidence for how repeated oral co-exposure may influence toxic responses was available for only 11 pairs. The evidence for interactions was adequate to suggest possible influences on the critical-effect toxicity of barium and calcium (kidney effects), manganese (neurotoxic effects), sodium (cardiovascular effects), and strontium (skeletal effects).

Hazard index approaches for exposure to mixtures with the subject metals and a hazard quotient for manganese should be accompanied with qualitative statements about the likely susceptibility of iron-deficient individuals to manganese neurotoxicity, the possible joint toxic action of excess iron and excess manganese on neurological endpoints, and the possible, but uncertain, protective effects of concurrent exposure to excess calcium or magnesium. Calculation of a neurological target toxicity hazard index with hazard quotients for barium and manganese should be accompanied by qualitative statements that: (1) available interaction data for barium and manganese are inadequate to assess whether the joint action of these metals on neurotoxic endpoints may be dose-additive, greater-than-dose-additive, or less-than-dose-additive; and (2) accumulation of iron, manganese, and other metals in the brain may jointly act to produce neurological impairment that may not be accounted for in a neurological hazard index based only on barium and manganese.

Hazard index approaches using a hazard quotient for sodium-induced hypertension should be accompanied with qualitative statements about the possible, but uncertain, protective actions of concomitant high exposure levels to calcium and magnesium against sodium-induced hypertension. Calculation of a cardiovascular hazard index with hazard quotients for barium and sodium should be accompanied by qualitative statements that available interaction data for barium and sodium are inadequate to assess whether the joint action of these metals to produce cardiovascular effects may be dose-additive, greater-than-dose-additive, or less-than-dose-additive and that possible contributions to effects on blood pressure from excess iron are not accounted for in the hazard index due to the lack of adequate data for TTD development.

Hazard index approaches utilizing a hazard quotient for adverse skeletal effects from strontium should be accompanied by qualitative statements about: (1) the uncertainty that excess calcium may counteract the development of strontium-induced skeletal effects and (2) skeletal effects from excess sodium are also possible, but available data are inadequate for TTD development. The hazard quotient for adverse skeletal effects from strontium should also be accompanied with a qualitative statement about the potential beneficial effects of strontium in inhibiting bone resorption and stimulating bone formation in osteoporotic animals and humans presumably via interactions with the CaSR.

Hazard index approaches utilizing a hazard quotient for calcium based on kidney stone formation should be accompanied by qualitative statements of the uncertainties associated with calcium's potential to induce kidney stones in humans and magnesium's potential to protect against kidney stone formation in humans. Calculation of a kidney hazard index with hazard quotients for barium, calcium, and magnesium should be accompanied by qualitative statements that available interaction data for barium, calcium, and magnesium are inadequate to assess whether the joint toxic action may be dose-additive, greater-than-dose-additive, or less-than-dose-additive and that possible contributions to kidney adverse effects from excess iron and excess sodium would not be captured in the hazard index due to inadequate data for kidney TTDs for these metallic cations.

Chapter 5

QUESTION: Are you aware of any additional references that should be included? Please provide citations.

COMMENT 17: They are included above.

RESPONSE: *As noted in the Response to Comment 9, discussion of some of the suggested articles were added to Sections 2.1 and 2.2 and Chapters 3 and 4.*

QUESTION: Please note the suggested location in text that new articles should be cited.

COMMENT 18: Chapter 2.

RESPONSE: *As noted in the Response to Comment 9, discussion of some of the suggested articles has been added to Sections 2.1 and 2.2 and Chapters 3 and 4.*

Appendices A through I

Please provide comments on the content and presentation of each of the included appendices.

COMMENT 19: The Appendices are adequate.

RESPONSE: *No response needed.*