National Toxicology Program
U.S. Department of Health and Human Services

# Protocol for Systematic Review of Effects of Fluoride Exposure on Neurodevelopment

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Office of Health Assessment and Translation
Division of the National Toxicology Program
National Institute of Environmental Health Sciences

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# BACKGROUND

## Exposure

Fluoride, a naturally occurring element, is used for the prevention of dental caries. Humans are exposed to fluoride via dental products (e.g., toothpaste, mouth rinses, supplements) and fluoride-supplemented drinking water. Fluoride also can occur naturally in drinking water. Other sources of human exposure include foods, beverages, industrial emissions, pharmaceuticals, and pesticides (e.g., cryolite, sulfuryl fluoride). Soil ingestion is another source of fluoride exposure in young children (EPA 2010).

## Water Fluoridation

The U.S. Public Health Service (PHS) first recommended communities add fluoride to drinking water in 1962. PHS guidance is advisory, not regulatory, which means that, while PHS recommends community water fluoridation as an effective public health intervention, the decision to fluoridate water systems is made by state and local governments. For community water systems that add fluoride, PHS now recommends a fluoride concentration of 0.7 milligrams/liter (mg/L).1 Under the Safe Drinking Water Act, the U.S. Environmental Protection Agency (EPA) sets maximum exposure level standards for drinking water quality. For fluoride, the enforceable standard is set at 4.0 mg/L, to protect against skeletal fluorosis. A secondary drinking water standard of 2.0 mg/L protects against moderate to severe dental fluorosis. The secondary standard is not enforceable but requires systems to notify the public if the average levels exceed it. EPA is reviewing the current drinking water standards for fluoride (EPA 2013).

## Concerns for Potential Fluoride Toxicity

Controversy over community water fluoridation stems from concerns about the potential harmful effects of fluoride and the ethics of water fluoridation. The most commonly cited health concerns related to fluoride and water fluoridation are bone fractures and skeletal fluorosis, decreased intelligence quotient (IQ) and other neurological effects, cancer, and endocrine disruption. Effects on neurological function, endocrine function (e.g., thyroid, parathyroid, pineal), metabolic function (e.g., glucose metabolism), and carcinogenicity were assessed in the 2006 National Research Council (NRC) report Fluoride in Drinking Water: A Scientific Review of EPA's Standards (NRC 2006). The NRC review considered adverse effects of water fluoride, focusing on a range of concentrations (2–4 mg/L) above the current 0.7-mg/L recommendation for community water fluoridation (NRC 2006). At fluoride levels below 4.0 mg/L, NRC did not find sufficient evidence of negative health effects, other than severe dental fluorosis. The conclusions from the NRC review were the primary source of information for the potential hazard summary in a 2015 report by the U.S. Department of Health and Human Services (DHHS), Federal Panel on Community Water Fluoridation. The NRC report noted several challenges to evaluating the literature, including: deficiencies in reporting quality, consideration of all sources of fluoride exposure, consideration of potential confounding, selection of appropriate control subject populations in

[1]For many years, most fluoridated community water systems used fluoride concentrations ranging from 0.8 to 1.2 mg/L [US DHHS] U.S. Department of Health and Human Services Federal Panel on Community Water Fluoridation. 2015. PHS Recommendation for Fluoride Concentration in Drinking Water. Available at http://www.publichealthreports.org/documents/PHS_2015_Fluoride_Guidelines.pdf [accessed 17 September 2015]. Public Health Reports 130:318-331.

epidemiology studies, demonstrated clinical significance of endocrine effects, and the biological relationship between histological, biochemical, and molecular alterations with behavioral effects.

## Nominations to NTP

In 2015, the National Toxicology Program (NTP) received nominations from the public to conduct analyses of fluoride and developmental neurobehavioral toxicity, endocrine disruption, and cancer. NTP is moving forward with the consideration of the developmental neurobehavioral evidence. For cancer and endocrine disruption, NTP is analyzing the amount of evidence available and the merit of pursuing systematic reviews, given factors such as the extent of new research published since previous evaluations and whether these new reports address or correct the deficiencies noted in the literature (NRC 2006; OEHHA 2011; SCHER 2011).

Regarding neurotoxicity and neurobehavioral effects, the main conclusions in the 2006 NRC report were:

> "Animal and human studies of fluoride have been published reporting adverse cognitive and behavioral effects. A few epidemiologic studies of Chinese populations have reported IQ deficits in children exposed to fluoride at 2.5 to 4 mg/L in drinking water. Although the studies lacked sufficient detail for the committee to fully assess their quality and relevance to U.S. populations, the consistency of the results appears significant enough to warrant additional research on the effects of fluoride on intelligence." [p. 8] (NRC 2006)

> "A few animal studies have reported alterations in the behavior of rodents after treatment with fluoride, but the committee did not find the changes to be substantial in magnitude. More compelling were studies on molecular, cellular, and anatomical changes in the nervous system found after fluoride exposure, suggesting that functional changes could occur. These changes might be subtle or seen only under certain physiological or environmental conditions. More research is needed to clarify the effect of fluoride on brain chemistry and function." [p. 8] (NRC 2006)

Since the 2006 NRC report was released, 10+ epidemiological studies and 45+ experimental animal studies have been published addressing the potential neurobehavioral effects of fluoride. Recent reviews of the human literature suggest that high levels of naturally occurring fluoride in water (>1.5 parts per million [ppm]) could be associated with negative health effects, including lower IQ (Choi et al. 2012; Sutton et al. 2015). Overall, many of these studies were considered low quality, as they did not fully account for known confounding factors regarding IQ (e.g., nutritional status, socioeconomic status) or other potential factors influencing IQ (e.g., iodine deficiency, chemical contaminants in the ground water such as arsenic and lead) . Very few studies have assessed the association between fluoride levels relevant to community water fluoridation practices and neurobehavioral effects. Based primarily on an analysis of a prospective cohort study conducted in New Zealand (Broadbent et al. 2015), Sutton et al. (2015) concluded there was no evidence of an association with lowered IQ in studies of community water fluoridation.

NTP recently published a systematic review of the animal evidence on the effects of fluoride on learning and memory (NTP 2016). The systematic review found a low-to-moderate level of evidence that learning and memory deficits occur in experimental animals at fluoride concentrations greater than 0.7 ppm. The

evidence was strongest (moderate) in animals exposed as adults and evidence was weaker (low) in animals exposed during development. NTP is conducting additional studies to assess the effect of fluoride exposure on learning and memory. The results from the ongoing experimental animal work will be incorporated into the current review, which will consider the epidemiological, animal, and mechanistic evidence in its conclusions. The NTP review will also identify key research and data gaps for additional study.

# OVERALL OBJECTIVES AND SPECIFIC AIMS

The overall objective of this evaluation is to undertake a systematic review of the existing human, experimental animal (non-human mammals), and mechanistic studies to develop hazard identification conclusions about whether fluoride exposure is associated with neurobehavioral effects. The systematic review will be based on guidance outlined in the Office of Health Assessment and Translation (OHAT) Handbook for Conducting a Literature-Based Health Assessment (NTP 2015a).

## Specific Aims

- Identify epidemiological and experimental animal literature (extending the 2016 evaluation) reporting the effects of fluoride exposure on neurobehavioral outcomes, especially outcomes related to learning, memory, and intelligence following exposure during development. Effects on thyroid function will also be assessed to help evaluate potential mechanisms of impaired neurological function. Studies reporting in vitro and other types of mechanistic evidence relating to neurobehavioral outcomes or thyroid function also will be identified.

- Extract data on relevant health outcomes from included epidemiological and experimental animal studies. An iterative approach will be used to determine which in vitro studies are most important to extract or summarize, based on factors such as concentrations tested, directness, and relevance of the in vitro outcomes to the human and animal outcomes of interest.

- Assess risk-of-bias for individual epidemiological and experimental animal studies.

- Synthesize the evidence across studies that assessed learning and memory using a narrative approach or meta-analysis (if appropriate) and evaluate sources of heterogeneity.

- Use the GRADE (Grading of Recommendations Assessment, Development, and Evaluation) framework to rate confidence in the body of evidence for effects on learning and memory according to one of four statements: 1. High, 2. Moderate, 3. Low, or 4. Very Low/No Evidence Available.

- Translate confidence ratings into level of evidence for effects on learning and memory for human and animal bodies of evidence separately according to one of four statements: 1. High, 2. Moderate, 3. Low, or 4. Inadequate.

- Combine the level-of-evidence ratings for human and animal bodies of evidence and consider the degree of support from mechanistic data to reach one of five possible hazard

identification conclusions: Known, Presumed, Suspected, Not classifiable, or Not identified to be a hazard to humans.

- Characterize uncertainty based on describing limitations of the evidence base, limitations of the systematic review, and consideration of dose relevance and pharmacokinetic differences when extrapolating findings from animal studies to human exposure levels, and identify key data gaps and research needs.

## PECO Statement

A PECO statement (Participants/Population, Intervention/Exposure, Comparator, Outcome) was developed to address and understand the potential effects of fluoride on neurological and thyroid outcomes in humans, experimental animals, and in vitro model systems (Table 1). The PECO statement is used to help develop the specific research questions, search terms, and inclusion/exclusion criteria for the systematic review (J Higgins and S Green 2011).

**Table 1. PECO (Populations, Exposures, Comparators, Outcomes) Statement**

| PECO Element | Evidence |
|---|---|
| **Population** | **Human:** Epidemiological studies, with the exception of case studies and case reports. |
| | **Animal:** Non-human mammalian animal species (whole organism). |
| | **In vitro:** Human or animal cells, tissues, or biochemical reactions (e.g., ligand binding assays) with in vitro exposure regimens. |
| **Exposure** | Exposure to fluoride based on administered dose or concentration, biomonitoring data (e.g., urine, blood, other specimens), environmental measures (e.g., air, water levels), or job title or residence. Relevant forms are those used as additives for water fluoridation:<br>• Fluorosilicic acid (also called hydrofluorosilicate; CASRN 16961-83-4)<br>• Sodium hexafluorosilicate (also called disodium hexafluorosilicate or sodium fluorosilicate; CASRN 16893-85-9)<br>• Sodium fluoride (CASRN 7681-49-4)<br>• Other forms of fluoride that readily dissociate into free fluoride ions (e.g., potassium fluoride, calcium fluoride, ammonium fluoride)<br>• Aluminum fluoride or aluminum fluoride complexes |
| **Comparator** | **Human:** A comparison population exposed to lower levels of fluoride (e.g., exposure below detection levels) or no fluoride. |
| | **Animal and in vitro:** Exposed to vehicle-only treatment. |
| **Outcomes** | **Human and Animal:** Learning, memory, intelligence, other forms of cognitive behavior, other neurological outcomes (e.g., anxiety, aggression, motor activity), or biochemical changes in the brain, nervous system tissue. Also, measures of thyroid function, biochemical changes, or thyroid tissue. |
| | **In vitro:** Endpoints related to neurological and thyroid function, including neuronal electrophysiology; mRNA, gene, protein expression; cell proliferation or death in brain or thyroid tissue/cells; neuronal signaling; synaptogenesis, etc. |

# METHODS

## Step 1. Problem Formulation

NTP received a nomination from the public in June 2015 to conduct an analysis of fluoride developmental neurobehavioral toxicity. The PECO statement was developed to address this nomination and was presented to the NTP Board of Scientific Counselors during its December 1-2, 2015 meeting.

## Step 2. Search and Select Studies for Inclusion

### *Searching electronic databases*

Database search strategies were developed using index terms and text words based on key elements of the PECO Statement. The following databases will be searched (full details of the search strategies are presented in Appendix 1):

- BIOSIS (Thomson Reuters)
- EMBASE (Elsevier)
- PsycINFO (APA PsycNet)
- PubMed (NLM)
- Scopus (Elsevier)
- Web of Science (Thomson Reuters; Web of Science indexes the journal Fluoride)

Searches will not be restricted by publication date. No language restrictions will be applied.

### *Searching other resources*

Reference lists of included studies from the full-text literature screen, reference lists of studies that do not contain original data (i.e., reviews, editorials, commentaries), and the Fluoride Action Network website will be searched for additional relevant publications.

### *Selection criteria for the evidence*

Studies will be screened for inclusion using a structured form in SWIFT-Active Screener, a machine-learning software program used to priority-rank studies for screening. SWIFT-Active Screener employs active learning to incorporate user feedback during the screening process to refine a statistical model that continually ranks the remaining studies according to their likelihood for inclusion. In addition, the software includes a statistical algorithm to estimate predicted recall (percent of truly relevant studies identified) while users work, thus providing a statistical basis for a decision about when to stop screening (Miller et al. 2016). The title and abstract screen will be stopped once the statistical algorithm in SWIFT-Active Screener estimates ≥98% predicted recall. Two members of the evaluation design team will independently conduct a title and abstract screen of the search results to identify studies that meet the eligibility criteria. For citations with no abstract or non-English abstracts, articles will be screened based on title relevance (title should indicate clear relevance), page numbers (articles less than ≤2 pages long will be assumed to be conference reports, editorials, or letters), and/or PubMed MeSH headings.

Studies that are not excluded based on the title and abstract will advance to the full-text review. Full-text copies of potentially relevant articles will be screened for inclusion using a structured form in DistillerSR (Evidence Partners; https://www.evidencepartners.com/products/distillersr-systematic-review-software/) by two independent reviewers. When results between reviewers disagree, the two reviewers will discuss discrepancies and consult with technical advisor(s) if necessary to decide on the status (include/exclude) of each discrepancy. Translation assistance will be sought to assess the relevance of non-English studies. In addition, full-text copies of potentially relevant review articles also will be screened by two reviewers to identify studies from the review reference lists that satisfy the inclusion criteria.

To be eligible for inclusion, studies must comply with the PECO criteria (Table 1). Studies that do not meet the PECO criteria will be excluded. In addition to the PECO criteria, the following exclusion criteria will apply:

- Records that do not contain original data but are relevant to the PECO statement, such as reviews, editorials, or commentaries. Reference lists from these materials, however, will be reviewed to identify potentially relevant studies not identified from the database searches. These studies will be assessed for eligibility for inclusion based on the process described above.

- Conference abstracts or reports. Attempts will be made, however, to contact authors of recent conference abstracts (~past 2–3 years) to assess publication status when a published version of the full study was not identified via the database search.

NTP includes only publicly accessible information in its evaluations. This information is typically based on studies published in peer-reviewed journals. NTP, however, can consider unpublished data or data presented in the grey literature (e.g., conference reports, theses/dissertations, technical reports, white papers) that have not undergone peer review, provided the owners of the data are willing to have the study details and results made publicly accessible. NTP would organize a peer review of any submitted unpublished data (NTP 2015a).

The list of included and excluded studies will be posted at the NTP website for this project once screening is completed. The results of the literature search will be presented in a PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram (Moher et al. 2009).

## Step 3. Data Extraction and Content Management

Data will be extracted from individual studies by members of the evaluation team. Data extraction and warehousing will be carried out using Health Assessment Workspace Collaborative (HAWC), a free and open-source, web-based software application.[2] Data extraction elements collected from epidemiological studies are listed in Appendix 2, from animal studies in Appendix 3, and in vitro studies in Appendix 4. The content of the data extraction might be revised following the identification of the studies included in the review. The data extraction results for included studies will be presented in the technical report and the data extraction results will be available for download from HAWC in Excel format when the project is completed. Data extraction will be performed by one member of the evaluation team and checked by one other member. Any discrepancies in data extraction will be resolved by discussion or

[2]Health Assessment Workspace Collaborative (HAWC): A Modular Web-based Interface to Facilitate Development of Human Health Assessments of Chemicals. https://hawcproject.org/portal/.

consultation with a third member of the evaluation team. Missing data from individual animal and in vitro studies generally will not be sought. More attempts, however, will be made for missing data from an otherwise well-reported and well-conducted study, such as missing group size or variance descriptor (standard deviation/standard error from certain animal studies). Routine attempts will be made to obtain missing information from epidemiological studies. Outreach to study authors will be recorded in HAWC as unsuccessful if researchers do not respond to an email or phone request within 1 month of the attempt to contact. Missing information to assess risk-of-bias for animal and epidemiological studies will be sought routinely (see below).

### *Standardizing results from behavioral tests and dose levels in experimental animal studies*

Results from behavioral tests will be transformed, when possible, to a common metric of percent change from control response to help assess dissimilar but related outcomes measured with different scales. In this project, percent control response will be used as the common metric because it is recommended for assessing dissimilar but related outcomes measured with different scales (Vesterinen et al. 2014). Percent control group calculations will be based on sample size, means, and standard deviation or standard error values presented in the studies.

For studies in which experimental animals were dosed with sodium fluoride (NaF) or other forms of dissociable fluoride, dose levels will be converted to fluoride equivalents (F), for example, 100 ppm NaF = 45.3 ppm (mg/L) fluoride. In studies where F was administered directly (often reported simply as "fluoride"), no such conversions are conducted. Fluoride dose levels are standardized to mg/kg-d and ppm (mg/L). Conversions will be made using water consumption rates and body weights for rats and mice reported in the EPA dosimetry (US EPA 1988; 1994). In each case, the "subchronic" values will be used because this period fit the maternal or single-generation dosing periods in most studies. The strain-specific and sex-specific values will be used when available; for strains that are not available, the "other" values will be used. For studies in which dosing is through the feed, the first conversion is from food ppm to mg/kg-d. Then, the "effective water concentration" is estimated by multiplying the converted dietary dose by body weight and dividing by water consumption rate. The uncertainty in these estimates should be considered higher than in water consumption studies.

Unless otherwise reported by study authors, a fluoride background level of 0 ppm (0 mg/kg-d) will be assumed in experimental animal studies. As available, dose levels will be presented as mg/kg-d and ppm. Dose conversions using US EPA (1988, 1994) default food or water consumption rates and body weights will be performed for any studies not reporting dose levels as mg/kg-d. Importantly, dose levels in mg/kg-d can vary for a given ppm across different studies if the studies use different species, strains, or sexes of animals that are assumed to have different food or water consumption rates.

## Step 4. Quality Assessment of Individual Studies

Risk-of-bias (RoB) will be assessed for individual studies using a tool developed by OHAT that takes a parallel approach for evaluating RoB from human and animal studies in order to facilitate consideration of RoB across evidence streams with common terms and categories (NTP 2015a) (Table 2). The RoB tool consists of a set of 11 questions that are answered based on the specific details of individual studies to develop RoB ratings (using the four options in Table 2) for each question. The subset of questions that will be used to assess RoB for an individual study is based on the study design (Table 2); specific protocols have been developed for this systematic review based on evidence stream and the type of human study design (Appendix 5). For example, the subset of RoB questions applicable to all experimental study designs includes a question on randomization of exposure that would not be

applicable to observational study designs. RoB will be assessed at the outcome level because study design or method specifics might increase the RoB for some outcomes and not for others within the same study. Missing information to assess RoB for human and animal studies will be routinely sought. Outreach to study authors will be recorded in HAWC as unsuccessful if researchers do not respond to an email or phone request within 1 month of the attempt to contact. Any information not reported will be assumed as not having been conducted (e.g., randomization, blinding), resulting in an assessment of "probably high" RoB.

For both epidemiological and experimental animal studies, two reviewers will independently conduct RoB evaluations and reach consensus on disagreements by discussion and consultation with technical expert(s) as needed. Assessors will be trained using the criteria in Appendix 5 with an initial pilot phase undertaken to improve clarity of criteria that distinguish between adjacent ratings and to improve consistency among assessors. All team members involved in the RoB assessment will be trained on the same set of studies and asked to identify potential ambiguities in the criteria used to assign ratings for each question. Any ambiguities and rating conflicts will be discussed relative to opportunities to refine the criteria to distinguish more clearly between adjacent ratings. If major changes to the RoB criteria are made based on the pilot phase (i.e., those that would likely result in revision of response), they will be documented in a protocol amendment along with the date of modifications and the logic for the changes. Information about confounding, exposure characterization, outcome assessment, and other important issues might be identified during or after data extraction, which can lead to further refinement of the RoB criteria (Sterne et al. 2014). After assessors have independently made RoB determinations for a study across all RoB questions, the two assessors will compare their results to identify discrepancies and attempt to resolve them. Any remaining discrepancies will be considered by the project lead and, if needed, other members of the evaluation design team and technical advisors. The final RoB rating for each question will be recorded with a statement of its basis.

**Table 2. Response Options for Each RoB Question**

| | |
|---|---|
| + | ***Definitely Low*** *risk-of-bias*:<br>Direct evidence of low risk-of-bias practices<br>(Could include specific examples of relevant low risk-of-bias practices) |
| + | ***Probably Low*** *risk-of-bias*:<br>Indirect evidence of low risk-of-bias practices **OR** deviations from low risk-of-bias practices for these criteria during the study are deemed not to bias results appreciably, including consideration of direction and magnitude of bias |
| – N | ***Probably High*** *risk-of-bias*:<br>Indirect evidence of high risk-of-bias practices **OR** insufficient information (e.g., not reported or "NR") is provided about relevant risk-of-bias practices |
| – | ***Definitely High*** *risk-of-bias*:<br>Direct evidence of high risk-of-bias practices<br>(Could include specific examples of relevant high risk-of-bias practices) |

### *Critical risk-of-bias domains for epidemiology studies*

Confidence in exposure or exposure assessment, the study design accounting for confounding variables, and the confidence in the outcome assessment (including blinding of outcome assessors to subjects' exposure levels) are the critical risk-of-bias domains that will be used to evaluate the potential for an

overall very serious RoB for individual studies, referred to as a "tier 3" study in the OHAT Handbook (NTP 2015a). Studies considered "probably high" or "definitely high" RoB in several of these domains are considered to pose an overall very serious RoB. These studies might be excluded from the analysis when they represent a sizeable portion of the studies considered for evidence integration.

### *Rationale for critical risk-of-bias domains for human studies*

- Differential or non-differential misclassification of the outcome through an improper definition of the outcome status or errors at the data collection stage may lead to an over- or underestimation of the effect size (Szklo 2014). Confidence in the outcome assessment for observational epidemiology studies will be evaluated both in terms of the specific measurement instruments used and with regard to the steps taken towards blinding the assessment of the outcome. Ideally, epidemiologic studies would include independent assessments of outcome measure validity both in the population for which it was originally designed, and with modifications appropriate to the study population of interest (Sabanathan et al. 2015). However, studies utilizing well-documented tests with modification for the population being studied would be considered at least "probably low RoB", even without specific validity measures provided. Importantly, a validated outcome assessment instrument may still result in bias if the test assessors are not appropriately blinded to the exposure status. For this reason, failure to provide evidence of blinding at outcome assessment, scoring, and evaluation will be weighed more heavily than the specific outcome assessment measure. In cases where blinding is not possible due to discrete study populations and/or exposure locations, studies should be considered "probably high RoB" unless specific direct or indirect evidence of blinding is provided or steps were taken to minimize the potential bias due to lack of blinding.

- Confirmation of exposure is vital to proper analysis and effect assessment. This should include evidence of consistent assessment methods used throughout the study, detection and quantification limits, and the utilization of well-established methods that directly characterize the exposure or intake. Studies that do not measure individual exposures (that is, that use summary statistics for a given population or group), generally will rate probably or definitely high on exposure assessment risk of bias. Studies where summary statistics are poorly documented with regard to variability or range, numbers of samples from which estimates were derived, and source and timing of measurements, may be assigned definitely high RoB. Studies that measure or estimate individual exposures, biomarker levels (such as urinary fluoride), or fluoride intake will generally be assigned probably or definitely low RoB with regard to exposure assessment. Where non-water sources of fluoride are unlikely to contribute substantially to overall intake, using indirect measures of exposure such as drinking water levels will not, by itself, be sufficient grounds for increasing the risk of bias rating.

- In assessing the effect of an exposure on a given outcome, improper adjustment for confounders can bias the results towards or away from the null (Szklo 2014). Therefore, direct evidence should be provided that adjustments and/or considerations were made for any covariates that are known to effect the relationship between the exposure and outcome of interest in each study. For neurodevelopmental effects of fluoride exposure, key covariates include co-exposure to other chemicals associated with neurotoxicity (e.g., arsenic and lead) and iodine sufficiency. Failure to consider the distribution of the key covariates across the exposure groups will result in a "probably high RoB" or "definitely high RoB",

depending on the likelihood of those factors affecting the results of the final analyses. Furthermore, individual and parental demographic, socioeconomic, and health characteristics, nutrition and growth factors, parental IQ, and smoking and smoke exposure, and dental and skeletal fluorosis should all be given either direct or indirect consideration. Dental and skeletal fluorosis are highly correlated with fluoride exposure, so careful consideration should be given to how they are handled in the study, since such physical anomalies may impact performance on neurodevelopmental testing independent of fluoride exposure (von Hilsheimer and Kurko 1979). To receive a "definitely low RoB" rating, it will be necessary that studies both provide quantitative summaries of covariate values across exposure groups or the study population, and adjust for covariates in statistical analyses.

### *Critical risk-of-bias domains for animal studies*

Randomization to treatment group, confidence in outcome assessment (including blinding of the outcome assessors), adequate characterization of the administered chemical, and controlling for litter effects in developmental studies are considered key drivers in determining potential for an overall very serious RoB for individual studies, referred to as a "tier 3" study in the OHAT Handbook (NTP 2015a). Studies considered "probably high" or "definitely high" RoB in several of these domains are considered to pose an overall very serious RoB. These studies might be excluded from the analysis, although sensitivity analyses will be conducted to assess the impact of excluding studies on conclusions. Studies also might be considered tier 3 due to a combination of concern for RoB in a critical domain(s) and very poor reporting quality (e.g., not reporting the number of animals treated, species).

### *Rationale for critical risk-of-bias domains for animal studies*

- A lack of randomization can bias results away from the null toward larger effect sizes. This effect has been empirically assessed in both controlled human trials (reviewed in Higgins et al. 2011) and experimental animals (reviewed in Krauth et al. 2013). This element is widely recommended to assess RoB for controlled human trials (IOM 2011; Guyatt et al. 2011a; Higgins et al. 2011; Viswanathan et al. 2012) and is included in most RoB instruments for animal studies, reviewed in (Hooijmans et al. 2014; Krauth et al. 2013).

- A lack of blinding in randomized human subject trials has been shown empirically to be associated with larger estimations of intervention effects (on average a 9% increase in an odds ratio) (Pildal et al. 2007). Schulz et al. (1995) analyzed 250 controlled trials and found a 17% larger estimation of treatment effect, on average, in studies that were not double-blinded. In trials with more subjective outcomes, lack of blinding was associated with greater bias (Wood et al. 2008), indicating a greater impact with subjective evaluations of outcomes. A similar association between lack of blinding at outcome assessment and larger measures of effect has been reported for experimental animal studies (Bebarta et al. 2003; Sena et al. 2007; Vesterinen et al. 2010). Research specifically evaluating the impact of lack of blinding during allocation to treatment groups or during the course of the study in experimental animal studies is absent or minimal (NTP 2015b). In addition, concealment of animal dose information is problematic if exposure results in obvious effects on the normal daily functioning of the animal. Additional steps can be taken in experimental animal studies to reduce the RoB such as counterbalancing critical factors (e.g., sex, observers, apparatus, session, necropsy order) to equally distribute each factor across dose groups for endpoint assessments. Concern for lack of blinding during allocation or the conduct of the study can be attenuated if blinding was implemented at outcome assessment. For these reasons,

blinding at outcome assessment was weighed more heavily during RoB assessment than blinding during allocation concealment or during the course of the study. In neurobehavioral studies, concern for lack of blinding at outcome assessment is attenuated if behavioral parameters are measured by an automated, computer-driven system.

- In experimental animal studies, the confirmation of exposure and dose are important for exposure characterization but rarely empirically determined. Ideally, experimental animal studies would include independent verification of purity, dose level confirmation over the exposure period, and internal measure of the compound within the subject. Independent verification of purity would be considered best practice because the identity and purity as listed on the bottle can be inaccurate. Approximately 3% of commercially purchased chemicals are inaccurately labeled for the chemical, increasing to 10% when purity is considered (unpublished, personal communication Brad Collins, NTP chemist). Impurities also might be more toxic than the compound of interest. Studies that do not report source or purity will be considered "probably high RoB" for exposure, although if purity information is not reported but can be inferred from source (e.g., online product description), the rating would be "probably low RoB." Studies also will be considered "probably low RoB" for exposure if information on source and purity is not provided, but levels of fluoride measured in biological samples indicated a dose-response gradient across groups.

- In experimental animal studies, the preferred study design for developmental exposure is to consider the litter as the experimental unit for statistical analysis. Failure to adjust statistically or experimentally for litter in an animal study with developmental exposure or for a developmental endpoint is important. Animals generated from the same litter tend to respond more similarly than animals from different litters. The direction of the bias is away from the null toward a larger effect size (Haseman et al. 2001)[3] if the individual pup is considered as the statistical unit rather than the dam or litter from which the pup is derived. This can be due to inflation of the sample size or biological influence of the dam and litter.

### *Missing information for risk-of-bias assessment*

OHAT will attempt to contact authors of included studies by email to obtain missing information considered critical for evaluating risk-of-bias that cannot be inferred from the study. If additional information or data are received from study authors, risk-of-bias judgments will be modified to reflect the updated study information. If OHAT does not receive a response from the authors by one month of the contact attempt, a risk-of-bias response of "NR" for "not reported; probably high risk-of-bias" will be used and a note made in the data extraction files that an attempt to contact the authors was unsuccessful.

[3]In 2000, NTP cosponsored a workshop with EPA, "Low Dose Endocrine Disruptors Peer Review." As part of the peer review, a group of statisticians reanalyzed several "low" dose studies Haseman JK, Bailer AJ, Kodell RL, Morris R, Portier K. 2001. Statistical issues in the analysis of low-dose endocrine disruptor data. Toxicological sciences : an official journal of the Society of Toxicology 61:201-210. Based on studies that used littermates, they determined that litter or dam effects generally were present such that pups within a litter were found to respond more similarly than pups from different litters. The overall conclusion was "[f]ailure to adjust for litter effects (e.g., to regard littermates as independent observations and thus the individual pup as the experimental unit) can greatly exaggerate the statistical significance of experimental findings."

| Table 3. OHAT Risk-of-bias Tool | | | | | | |
|---|---|---|---|---|---|---|
| **Bias Domains and Questions** | **Experimental Animal[1]** | **Human Controlled Trials[2]** | **Cohort** | **Case-control** | **Cross-sectional[3]** | **Case Series** |
| **Selection Bias** | | | | | | |
| 1. Was administered dose or exposure level adequately randomized? | X | X | | | | |
| 2. Was allocation to study groups adequately concealed? | X | X | | | | |
| 3. Did selection of study participants result in appropriate comparison groups? | | | X | X | X | |
| **Confounding Bias** | | | | | | |
| 4. Did the study design or analysis account for important confounding and modifying variables? | | | X | X | X | X |
| **Performance Bias** | | | | | | |
| 5. Were experimental conditions identical across study groups? | X | | | | | |
| 6. Were the research personnel and human subjects blinded to the study group during the study? | X | X | | | | |
| **Attrition/Exclusion Bias** | | | | | | |
| 7. Were outcome data complete without attrition or exclusion from analysis? | X | X | X | X | X | |
| **Detection Bias** | | | | | | |
| 8. Can we be confident in the exposure characterization? | X | X | X | X | X | X |
| 9. Can we be confident in the outcome assessment? | X | X | X | X | X | X |
| **Selective Reporting Bias** | | | | | | |
| 10. Were all measured outcomes reported? | X | X | X | X | X | X |
| **Other Sources of Bias** | | | | | | |
| 11. Were there no other potential threats to internal validity (e.g., statistical methods were appropriate and researchers adhered to the study protocol)? | X | X | X | X | X | X |

[1]Experimental animal studies are controlled exposure studies. Non-human animal observational studies could be evaluated using the design features of observational human studies such as cross-sectional study design.
[2]Human Controlled Trials (HCTs): studies in humans with a controlled exposure, including Randomized Controlled Trials (RCTs) and non-randomized experimental studies.
[3]Cross-sectional studies include population surveys with individual data (e.g., NHANES) and population surveys with aggregate data (i.e., ecological studies).

## Step 5. Assessment of Confidence in the Body of Evidence

The quality of evidence for each outcome will be graded using the GRADE system for rating the confidence in the body of evidence (NTP 2015a; Guyatt et al. 2011a; Guyatt et al. 2011b; Guyatt et al. 2011c; Guyatt et al. 2011d). To approach evidence assessment, the framework provides guidance for determining overall certainty in the evidence as "high," "moderate," "low," or "very low" based on five factors for downgrading (e.g., RoB across studies, indirectness, unexplained inconsistency, imprecision, publication bias) and three for upgrading (e.g., large magnitude of the effect, dose response, plausible confounding). OHAT also considers consistency of findings across studies as a potential upgrade factor (Figure 1 and Table 4). More detailed guidance is available in the OHAT Handbook for conducting systematic review (NTP 2015a).

**Figure 1. Assessing Confidence in the Body of Evidence**

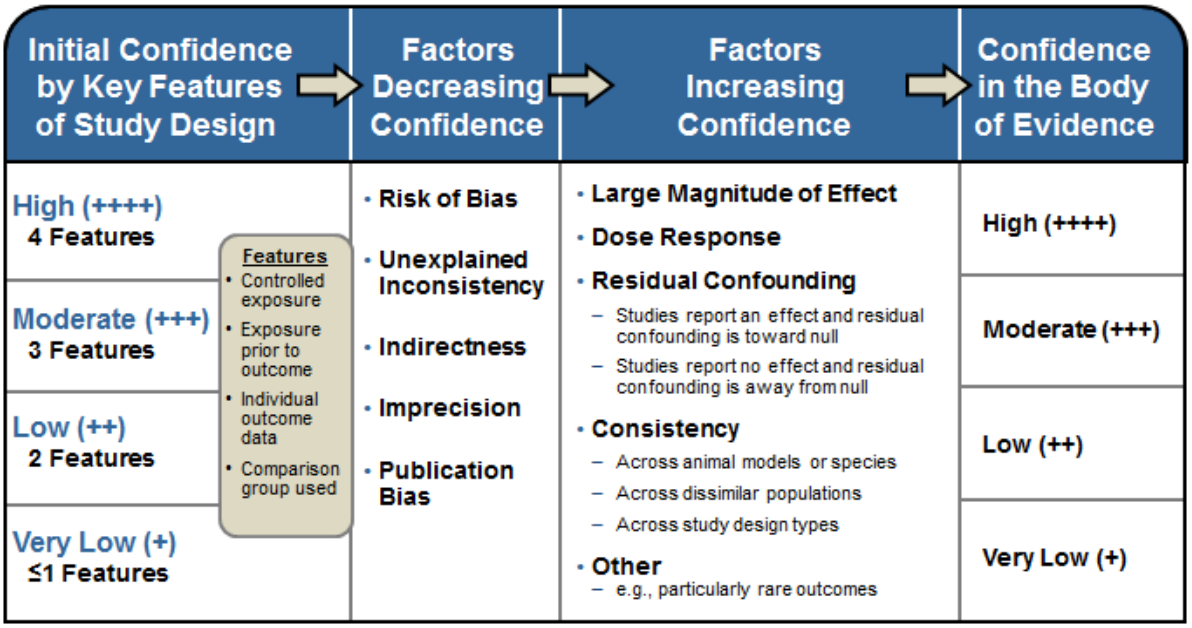



**Table 4. Key Factors when Considering Whether to Downgrade or Upgrade Across a Body of Evidence**

| Downgrade factors | Rationale for potential downgrade |
|---|---|
| **Risk-of-bias** | Risk-of-bias across all domains.<br>• **Human studies:** Critical factors include confounding bias, selection bias, exposure assessment, and outcome assessment.<br>• **Animal studies:** Critical factors include randomization, blinding at outcome assessment, exposure characterization (e.g., reporting source, purity, internal dose level), and control for litter effects (developmental exposure studies). |

| **Table 4. Key Factors when Considering Whether to Downgrade or Upgrade Across a Body of Evidence** | |
|---|---|
| **Downgrade factors** | **Rationale for potential downgrade** |
| **Unexplained inconsistency** | Focuses on the presence of unexplained inconsistency in studies of similar population (or experimental model system) and design. Inconsistency can be determined by assessing similarity of point estimates and extent of overlap between confidence intervals or more formally through statistical tests of heterogeneity. Sensitivity analysis can be used to assess the impact of specific variables on the outcome (e.g., variation in RoB at individual study level, species, route of dosing, statistical power). Inconsistency that can be plausibly explained is typically not used to support for a downgrade. If only one study is available, a rating of "none" is used to characterize inconsistency. |
| **Indirectness** | • **Human studies**: All exposure levels and scenarios encountered in human studies (e.g., general population, occupational settings) are considered direct and not downgraded.<br>• **Animal studies:**<br>– *Within animal models:* Are the reported endpoints direct indicators of learning and memory? Can factors that might impact an animal's performance in learning and memory tests, such as impaired motor or sensory function, be ruled out? Also consider the route of administration; oral is considered most relevant for fluoride.<br>– *Extrapolation between mammalian animals and humans:* In vivo mammalian model systems have demonstrated utility for examining autonomic, sensory, and motor system functioning as they relate to human health and are considered direct and not downgraded. Although human cognitive function is not easily assessed in such systems, aspects of learning and memory can be evaluated as based on learning theory that translates across species (Crawley 2007). Some neurobehavioral measures (e.g., social behaviors, aggression, risky behaviors), however, have not been demonstrated to translate readily between species, and other behaviors (e.g., verbal learning/performance, gender preferences) cannot be evaluated adequately in a non-human mammalian model system. Studies that report only these endpoints would be downgraded for lack of directness.<br>• **In vitro and non-mammalian animal studies:** Studies that involve direct treatment of cells or tissues or studies that only measure a biochemical reaction (e.g., receptor binding) are typically downgraded for lack of directness. |
| **Imprecision** | Confidence in quantitative measures such as effect sizes, identification of no observed effect level (NOEL) or lowest observed effect level (LOEL) doses, or parameters for benchmark dose analysis. Typically, 95% confidence intervals are used as the primary method to assess imprecision (Guyatt et al. 2011b). OHAT also considers whether studies are adequately powered when considering whether to downgrade. |
| **Publication bias** | Downgrade if "strongly detect" publication bias. Publication bias is difficult to assess, especially when multiple endpoints related to the primary outcome are reported in the same study, few studies are available, and papers do not report funding or conflicts of interest. Analytical tools, such as funnel plots or trim-and-fill approaches, can be used to assess publication bias but have substantial limitations and should be interpreted with caution (Guyatt et al. 2011c). |
| **Upgrade factors** | |

| Table 4. Key Factors when Considering Whether to Downgrade or Upgrade Across a Body of Evidence | |
|---|---|
| **Downgrade factors** | **Rationale for potential downgrade** |
| **Large magnitude of effect** | Factors to consider include the outcome being measured and the dose or exposure range assessed. |
| **Dose response** | Patterns of dose response are evaluated within and across studies. |
| **Consistency** | • **Human studies**: Consider whether consistent results were reported across populations that differ in factors such as geographic region, different measures of exposure, nature of the cohort, e.g., occupational, general population.<br>• **Animal studies:** Consider whether consistent results were reported in multiple experimental animal models or species. |

### *Evidence synthesis*

#### Endpoint grouping

Neurological endpoints will be broadly categorized using the frameworks below for human (Table 5) and animal (Table 6) studies. Evidence synthesis will focus on learning, memory, and intelligence. Studies reporting on other neurobehavioral endpoints will also be identified and summarized but not necessarily assessed for RoB at the individual study level (depending on the extent and nature of the literature).

| Table 5. Neurological Outcomes Grouping for Human Studies | | |
|---|---|---|
| **General Domain** | **Example Tests or Test Batteries** | **Example Endpoints or Subtests** |
| **Learning, Memory, Intelligence, Cognitive Development** | Neurobehavioral Core Test Battery (NCTB), Wechsler Intelligence Scale for Children (WISC) – Revised | Digit-Symbol Substitutions, Digit Span |
| | Wide Range Assessment of Memory and Learning (WRAML) | Verbal Memory Index, Visual Memory Index, Learning Index |
| | Wechsler Adult Intelligence Scale (WAIS) | Full Scale, Verbal, and Performance IQ |
| | Wechsler Preschool and Primary Scale of Intelligence (WPPSI), WPPSI-IV | Full Scale and Primary Index Scales (Verbal Comprehension Index, Working Memory Index, etc.) |
| | Halstead-Reitan Battery | Trail Making Test (Parts A and B) |
| | Wechsler Memory Scales (WMS) | Design Memory, Symbol Span |
| | Child and Adolescent Memory Profile (ChAMP) | Verbal Memory (lists), Visual Memory (objects) |
| | Stanford-Binet Test | Verbal and Non-Verbal Subtests in Visual Spatial Reasoning, Working Memory, etc. |
| | Raven's Progressive Matrices | |
| | Combined Raven's Test for Rural China | |

| | | |
|---|---|---|
| **Attention** | Neurobehavioral Evaluation System (NES) | Finger Tapping, Continuous Performance Test, Simple Reaction Time |
| | Neurobehavioral Core Test Battery (NCTB) | Simple Reaction Time |
| **Hyperactivity** | Behavioral Assessment System for Children, Child Behavior Checklist | Hyperactivity Symptoms, Attention Problems scale |
| **Motor/Sensory Function or Development** | Neurobehavioral Evaluation System (NES) | Simple Reaction Time, Hand-Eye Coordination |
| | Neurobehavioral Core Test Battery (NCTB) | Santa Ana, Aiming |
| | Brazelton Neonatal Behavioral Assessment Scale, NICU Network Neurobehavioral Scales | Reflexes, Startle Response, Habituation to Sensory/Light Stimuli, Hand-Mouth Coordination |
| | Nerve conduction velocity | Motor or Sensory Conduction Velocity |
| **Internalizing behaviors** | Beck Depression Inventory, Behavioral Assessment System for Children, Child Depression Inventory, SPENCE Child Anxiety Scale, State-Trait Anxiety Inventory | |
| | Neurobehavioral Evaluation System (NES) | Profile of Mood States |
| **Visual-Spatial or Visual-Motor Function** | Neurobehavioral Core Test Battery (NCTB) | Benton Visual Retention Test |
| | Wechsler Intelligence Scale for Children (WISC) – Revised or Wechsler Adult Intelligence Scale (WAIS) – Revised | Block Design, Digit Symbol Substitution |

**Table 6. Neurological Outcomes Grouping for Animal Studies**

| Endpoints | Example tests |
|---|---|
| **Learning and Memory** | Maze tests (Morris water maze, T-maze, Y-maze, Radial Arm); exploration (novel object recognition, mini-holeboard, activity cage); active and passive avoidance (step-down test, shuttle box); operant behavior |
| **Motor and Sensory Function** | Locomotor activity (open field, activity cage); movement coordination (akinesia/catalepsy, plank walking, rotarod, slanted surface, swim test); reflex (auditory startle, negative geotaxis, pain response: tail immersion and Von Frey hair test); developmental motor sensory landmarks (cliff avoidance, surface righting, pivoting/orienting reflex) |
| **Depression** | Forced swim; tail suspension test |
| **Anxiety** | Elevated plus maze |
| **Other** | Grooming; urination/defecation; sexual behavior; territorial behavior |

Considerations for pursuing a narrative or quantitative evidence synthesis

Heterogeneity within the available evidence will determine the type of evidence integration that is appropriate: either a quantitative synthesis (meta-analysis) or narrative approach for evidence integration. Where appropriate, a meta-analysis will be conducted to summarize the findings. Summaries of main study design characteristics for each included study will be compiled to determine comparability between studies, identify data transformations necessary to ensure comparability, and determine whether study heterogeneity is a concern. Including a study might not be appropriate when (1) data on exposure or outcome are too different to be combined, (2) concerns about high RoB are present, (3) endpoints or measurement scales are not sufficiently similar, or (4) other circumstances indicate that averaging study results would not produce meaningful results. Topic-specific experts will be consulted to help assess whether studies are too heterogeneous for meta-analysis to be appropriate. When quantitatively combining results is inappropriate or infeasible, findings will be narratively described or visually presented. The main characteristics considered when determining whether to combine studies quantitatively include the following for human studies and animal studies.

### *Human studies:*

- Study design (e.g., cohort, case-control study, cross-sectional, controlled trial, case report)
- Population demographics (sex, race/ethnicity, age or lifestage at exposure and outcome assessment)
- Exposure assessment method or matrix (e.g., blood, urine, hair, air, drinking water, job classification)
- Exposure range
- Neurobehavioral measurements, methodology, and scale
- Type of data (e.g., continuous, dichotomous), statistics presented in paper, ability to access raw or additional data
- Variation in degree of RoB at individual study level or very serious concern for RoB across studies

### *Animal studies:*

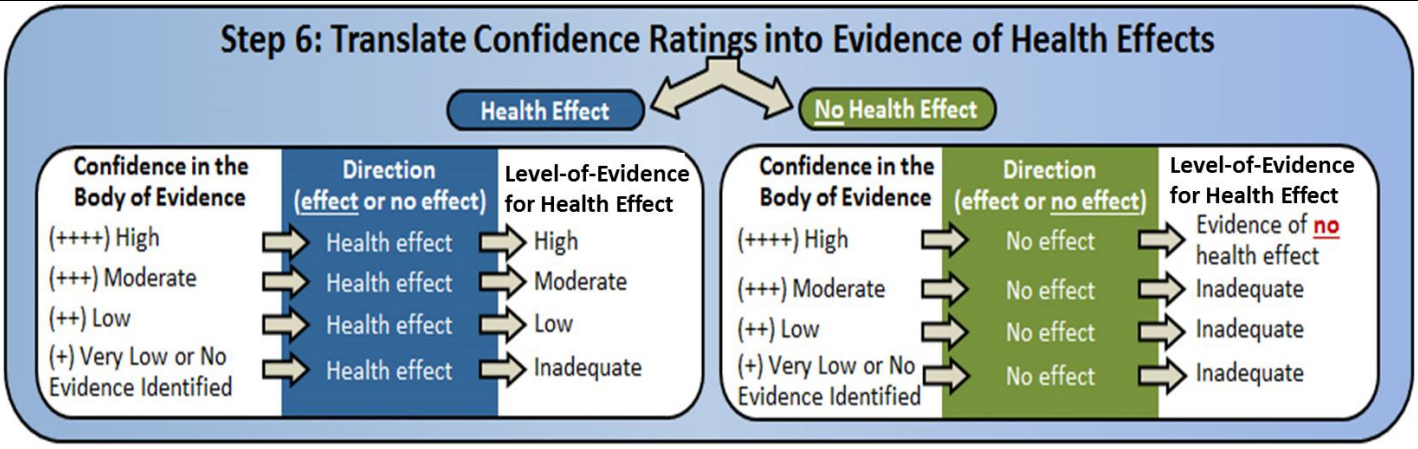
- Animal model used (species, strain, sex, genetic background)
- Age of animals (at start of treatment and outcome assessment, mating, pregnancy status)
- Dose levels, frequency of treatment, timing, duration, and exposure route
- Neurobehavioral measurements and methodology
- Type of data (e.g., continuous, dichotomous), statistics presented in paper, ability to access raw or additional data

- Variation in degree of RoB at individual study level or very serious concern for RoB across studies

## Step 6. Preparation of Level of Evidence Statement

The confidence in the body of evidence conclusions from Figure 1 will be translated into draft statements of health effects for human studies according to one of four statements: 1. High, 2. Moderate, 3. Low, or 4. Inadequate (Figure 2). The descriptor "evidence of no health effect" is used to indicate confidence that the substance is not associated with a health effect. Because of the inherent difficulty in proving a negative, the conclusion "evidence of no health effect " is only reached when there is high confidence in the body of evidence.

**Figure 2. Translate Confidence Ratings into Evidence of Health Effect Conclusions**




| Evidence Descriptors | Definition |
|---|---|
| **High Level-of-Evidence** | Confidence is high in the body of evidence for an association between exposure to the substance and the behavioral outcome(s). |
| **Moderate Level-of-Evidence** | Confidence is moderate in the body of evidence for an association between exposure to the substance and the behavioral outcome(s). |
| **Low Level-of-Evidence** | Confidence is low in the body of evidence for an association between exposure to the substance and the behavioral outcome(s). |
| **Evidence of No Health Effect** | Confidence is high in the body of evidence that exposure to the substance is not associated with the behavioral outcome(s). |
| **Inadequate Evidence** | Insufficient evidence is available to assess if the exposure to the substance is associated with the health outcome(s). |

## Step 7. Integrate Evidence to Develop Hazard Identification Conclusions

Initial hazard identification conclusions will be reached by integrating the highest level-of-evidence conclusion for neurodevelopmental effect(s) by integrating evidence of each outcome from the human and the animal evidence streams. Owing to ambiguities related to the interpretation of behavior tests in animals, it will likely not be possible to correlate specific outcomes in test animals with those in humans. Similarities in the general patterns of results for specific domains (such as learning and memory) may be considered across species as reviewers and experts consider appropriate, however. Human studies will provide the primary basis for hazard conclusions, with animal test results providing ancillary and supporting evidence.

Hazard identification conclusions may be reached on the groups of biologically related outcomes (using outcome groups identified in Table 5 and 6 as well as more specific endpoints if data are available to make more specific conclusions.

For similar/equivalent outcomes:

If the data support a specific neurological effect, the level-of-evidence conclusion for human data from Step 6 for that health outcome will be considered together with the level of evidence for the biologically related or equivalent non-human animal data to reach one of four initial hazard identification conclusions: Known, Presumed, Suspected, or Not classifiable. If either the human or animal evidence stream is characterized as "Inadequate Evidence," then conclusions are based on the remaining evidence stream alone (which is equivalent to treating the missing evidence stream as "Low" in Figure 3.)

For outcomes where the human and animal endpoints are dissimilar the hazard conclusion may be developed on either the human or animal evidence alone. As shown in Figure 3, if the level of confidence in a health effect is "High" in animals, but evidence is "Low" or "Inadequate" in humans, the overall level of evidence conclusion can be no greater than "Presumed." That is, animal evidence alone will not be sufficient to support a conclusion of "Known" neurodevelopmental effects in humans. If the human level of evidence rating of "Evidence of no health effect" from Step 6 is supported by a similar level of evidence rating for animal evidence for no health effect, the hazard identification conclusion would be "Not identified to be a hazard to humans."

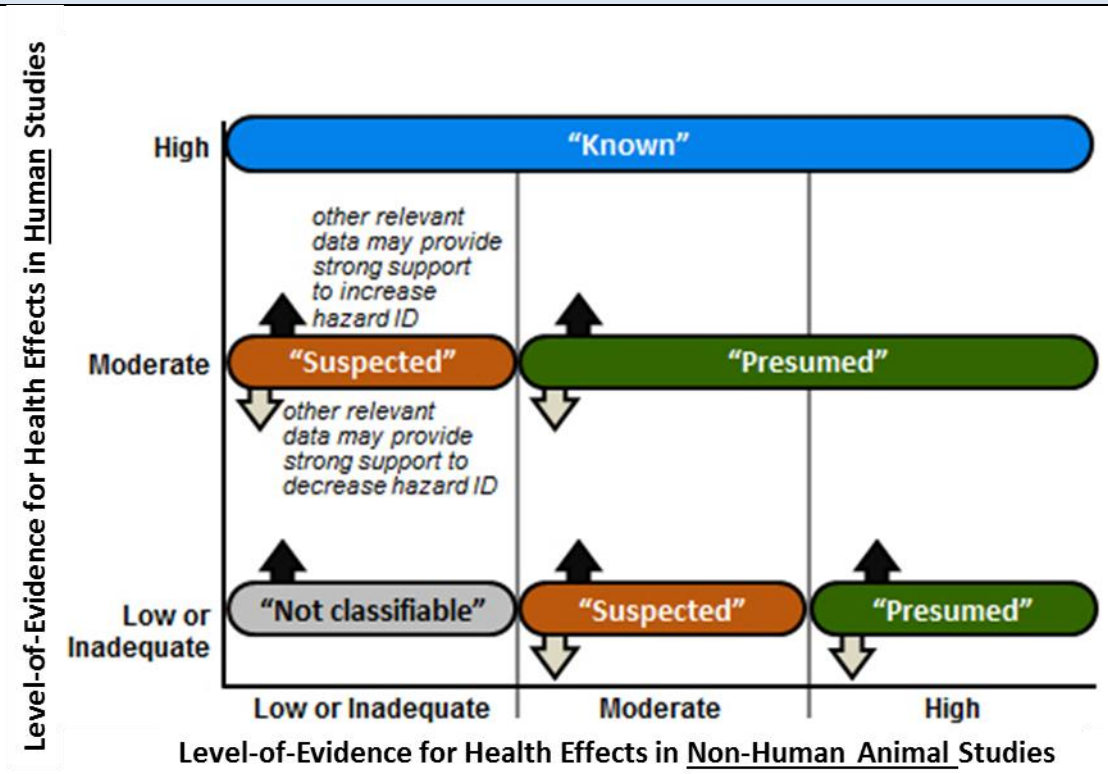
For similar/equivalent outcomes:

If the data support a specific neurological health effect, the level-of-evidence conclusion for human data from Step 6 for that health outcome will be considered together with the level of evidence for the biologically related or equivalent non-human animal data to reach one of four initial hazard identification conclusions: Known, Presumed, Suspected, or Not classifiable. If either the human or animal evidence stream is characterized as "Inadequate Evidence," then conclusions are based on the remaining evidence stream alone (which is equivalent to treating the missing evidence stream as "Low" in Figure 3.

For outcomes where the human and animal endpoints are dissimilar the hazard conclusion may be developed on either the human or animal evidence alone…

If the human level of evidence rating of "Evidence of no health effect" from Step 6 is supported by a similar level of evidence rating for animal evidence for no health effect, the hazard identification conclusion would be "Not identified to be a hazard to humans."

**Figure 3. Hazard Identification Scheme**



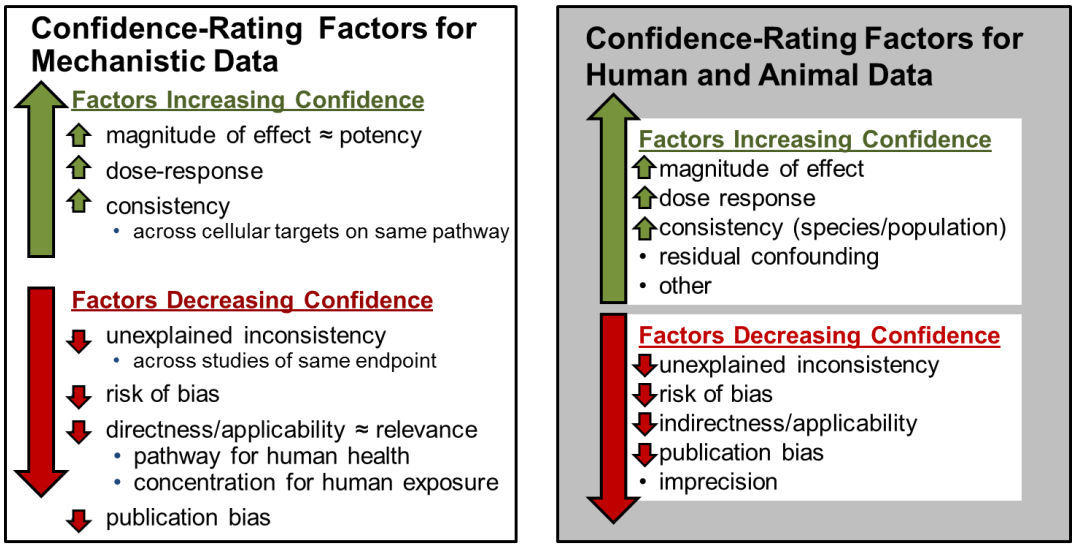

### *Consideration of mechanistic data*

NTP does not require mechanistic or mode-of-action data to reach hazard identification conclusions, although when available, this and other relevant supporting types of evidence may be used to raise (or lower) the category of the hazard identification conclusion. Mechanistic data can come from a wide variety of studies that are not intended to identify a disease phenotype. This source of experimental data includes in vitro and in vivo laboratory studies directed at cellular, biochemical, and molecular mechanisms that explain how a chemical produces particular adverse effects.

The strength of the support or opposition presented by the other relevant data is evaluated using the guidance presented in Figure 4 The factors outlined for increasing or decreasing confidence in that the mechanistic data support biological plausibility are conceptually similar to those used to rate confidence in bodies of evidence for human or animal in vivo studies. Evaluations of the strength of evidence provided by mechanistic data are made on an outcome-specific basis based on discussion by the evaluation team and consultation with technical advisors as needed.

The factors presented in Figures 3 and 4 will be considered in an iterative and effect-specific manner. For example, mechanistic data in animals may affect the level of evidence in animal studies (Figure 4), which can affect the overall hazard identification conclusion based on combined animal and human studies (Figure 3.) For example:

**Figure 4. Factors Considered in Evaluating the Support for Biological Plausibility**




- If mechanistic data provide strong support for biological plausibility of the relationship between exposure and the health effect, the hazard identification conclusion may be upgraded (indicated by black "up" arrows in the Step 7 graphic in Figure 3) from that initially derived by considering the human and non-human animal evidence together.

- If mechanistic data fail to provide support for biological plausibility of the relationship between exposure and the health effect, the hazard identification conclusion may be downgraded (indicated by gray "down" arrows in Figure 3) from that initially derived by considering the human and non-human animal evidence together.

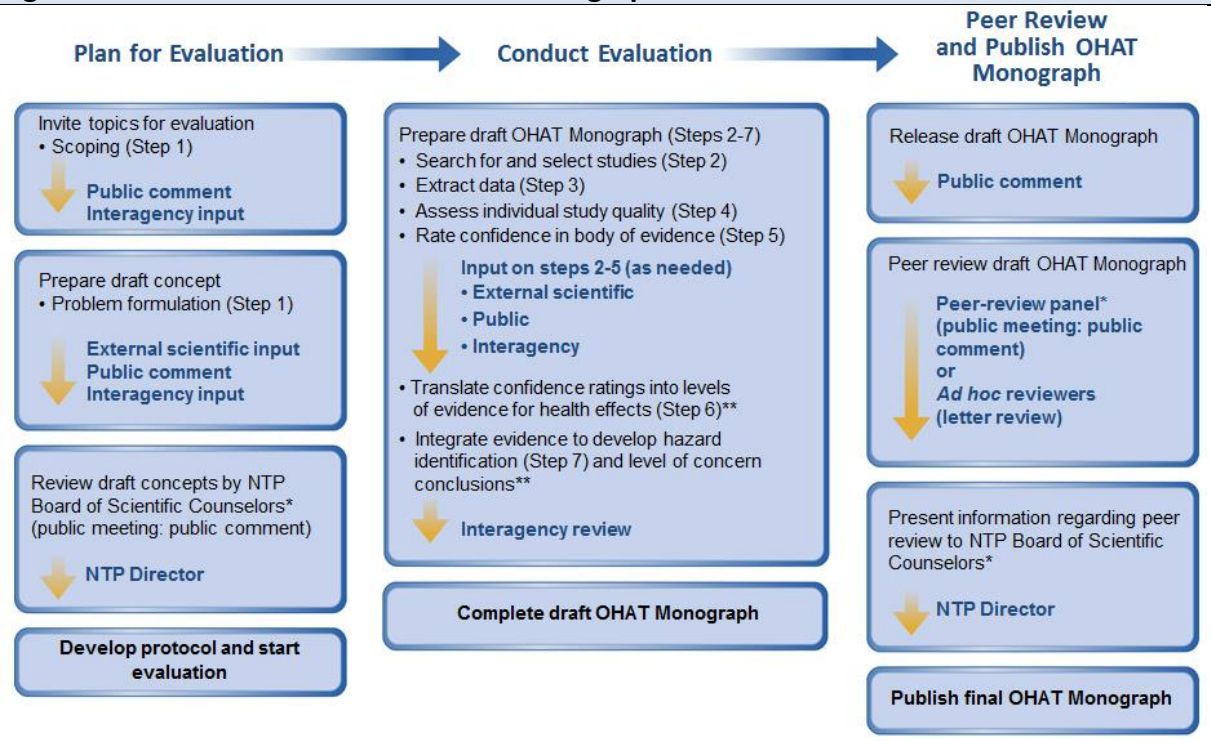
As mode of action pathways have not been well-established for the neurodevelopmental effects of fluoride, the primary role of mechanistic data will be to inform the biological plausibility of observed outcomes from in vivo data. That is, mechanistic data alone will not be sufficient by itself to support hazard identification conclusions for neurodevelopmental endpoints.

# NTP MONOGRAPH

## Evaluation Process

The problem formulation and evaluation process of preparing an NTP Monograph includes multiple opportunities for external scientific, public, and interagency inputs and external peer review (Figure 5).

**Figure 5. Evaluation Process for OHAT Monographs**




The use of systematic methods is in the evaluation, planning, and conduct phases and consists of Steps 1–7 (Rooney et al. 2014).
*Federally chartered advisory group
**Not included in state-of-science evaluation or expert panel workshop report

The NTP Monograph will include the methodology, results, discussion, and conclusion.

## Methodology

This section will provide a brief overview of the methodologies used in the review process, including:

- the research question (PECO statement);
- the search strategy used to identify and retrieve studies;
- the process for selecting the included studies;
- the quality assessment of included studies;
- the methods of data extraction;

- the methods used to critically appraise for RoB, sensitivity, and synthesize the data of included studies.

## Results

This section will include the results from the systematic review on the neurotoxicity of fluoride in human studies. Results will be presented in tables or figures as appropriate using HAWC. The results from the included studies will be discussed by outcome. This will include a description of:

- The number of studies identified considered relevant to PECO statement;
- The quality of the studies, as assessed using the appropriate tool;
- A data extraction and summary of the results from all studies;
- Quality of evidence and corresponding level of evidence conclusions rated according to one of four statements: 1. High, 2. Moderate, 3. Low, or 4. Very Low/No Evidence Available;
- Hazard identification conclusions based on integrating level of evidence ratings for human and animal data and consider the degree of support from mechanistic data (Known, Presumed, Suspected, Not classifiable, or Not identified to be a hazard to humans).

## Discussion

The discussion will provide a summary of the review findings and characterize uncertainty based on describing limitations of the evidence base, limitations of the systematic review, consideration of dose-relevance and pharmacokinetic differences when extrapolating findings from animal studies to human exposure levels, and identifying key data gaps and research needs.

## Conclusion

This will present the conclusion of the review.

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# ABOUT THE PROTOCOL

## Contributors

### *Evaluation Team*

Evaluation teams are composed of federal staff and contractor staff. Contractor staff members are screened for potential conflicts of interest. Federal staff members should do a self-evaluation. Epidemiologists and toxicologists on OHAT evaluation teams should have at least three years' experience and/or training in reviewing studies, including summarizing studies and critical review (e.g., assessing study quality and interpreting findings). Experience in evaluating occupational or environmental studies is preferred. Team members should have at least a master's degree or equivalent level of experience in epidemiology, toxicology, environmental health sciences, or a related field.

| Name | Affiliation |
|---|---|
| Kyla Taylor, PhD | NIEHS/NTP, Project Lead |
| John Bucher, PhD | NIEHS/NTP |
| Andrew Rooney, PhD | NIEHS/NTP |
| Vickie Walker | NIEHS/NTP |

### *Contract support*

Contractors listed below are anticipated to provide support necessary to complete the literature searches, study selection, data extraction, and risk of bias assessment

| Name | Affiliation |
|---|---|
| Anna Engstrom, PhD | ICF |
| Robyn Blain, PhD | ICF |
| Pam Hartman | ICF |
| William Mendez, PhD | ICF |
| Cara Henning, PhD | ICF |
| Johanna Rochester, PhD | ICF |
| Kristin Bornstein, PhD | ICF |
| Ali Goldstone | ICF |
| Canden Byrd | ICF |
| Anna Stamatogiannakis | ICF |
| Whitney Mitchell | ICF |
| Penelope Kellar | ICF |

### *Technical Advisors*

Technical advisors are outside experts retained on an as-needed basis to provide individual advice to the NTP for a specific topic. Technical advisors selected for this project were selected for their experience with fluoride exposure and neurotoxicity. Technical advisors were screened for conflict of interest prior to their service. Service as a technical advisor does not necessarily indicate that the advisor has read the entire protocol or endorses the final state-of-the-science document.

| Name | Affiliation |
|---|---|
| Joseph Braun, PhD | Brown University |
| Marie Sutton, PhD | Health Research Board |
| Thomas Zoeller, PhD | University of Massachusetts, Amherst |
| Thomas Webster, PhD | Boston University |
| Gail Wasserman, PhD | Columbia University |

### *Sources of Support*

National Institute of Environmental Health Sciences/Division of the National Toxicology Program

### *Protocol History and Revisions*

| Date | Activity or revision |
|---|---|
| **December 14, 2016** | **Draft evaluation protocol reviewed:** sent to technical advisors for comment/ review |
| **April 10, 2017** | **Draft human risk of bias protocol reviewed;** sent to technical advisors for comment/review |

| Date | Activity or revision |
|---|---|
| **May 2, 2017** | **Draft animal risk of bias protocol reviewed;** sent to technical advisors for comment/review |
| **June 2017** | **Draft finalized** |

# Appendix 1. ELECTRONIC DATABASE SEARCH STRATEGIES

***BIOSIS***

**Date of search: 11/28/2016; 6,743 results**

| **BIOSIS search terms** | | |
|---|---|---|
| #1 | Fluoride | **TOPIC**: ((fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) NOT (f-labeled OR "fluorine-18" OR radioligand* OR 18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan))) |
| #2 | Neurological and Thyroid outcomes | **TOPIC**: (Academic-performance OR active-avoidance OR ADHD OR alzheimer* OR amygdala OR antisocial OR anxiety OR anxious OR asperger* OR attention-deficit OR auditory OR autism OR autistic OR behavioral OR behaviors OR behavioural OR behaviours OR bipolar OR cerebellum OR cognition OR cognitive OR communication-disorder* OR comprehension OR cortical OR cranial OR delayed-development OR dementia OR dendrit* OR dentate-gyrus OR depression OR developmental-impairment OR Developmental-delay* OR developmental-disorder* OR dextrothyroxine OR diiodothyronine* OR diiodotyrosine OR down-syndrome OR dyslexia OR entorhinal-cortex OR epilep* OR euthyroid OR gait OR gangli* OR glia* OR gliogenesis OR goiter OR graves-disease OR hearing OR hippocamp* OR human-development OR hyperactiv* OR hyperthyroid* OR hypothalam* OR hypothyroid* OR impulse-control OR impulsiv* OR Intellectual-disability OR intelligence OR iodide-peroxidase OR IQ OR ischemi* OR language OR learning OR lewy-bod* OR locomotor OR long-term-potentiation OR long-term-synaptic-depression OR memory OR mental-deficiency OR mental-development OR mental-disorder* OR mental-illness OR mental-recall OR mental-deficit OR mobility OR monoiodotyrosine OR mood OR morris-maze OR morris-water OR motor-abilit* OR Motor-activities OR Motor-activity OR Motor-performance OR Motor-skill* OR Multiple-sclerosis OR myxedema OR nerve OR Nervous-system OR neural OR neurit* OR neurobehav* OR Neurocognitive-impairment OR neurodegenerat* OR Neurodevelopment* OR neurodisease* OR neurologic* OR neuromuscular OR neuron* OR neuropath* OR obsessive-compulsive OR OCD OR olfaction OR olfactory OR open-field-test OR optic OR palsy OR panic OR parahippocamp* OR paranoia OR paranoid OR parkinson* OR passive-avoidance OR perception OR perforant* OR personality OR phobia OR plasticity OR problem-solving OR proprioception OR psychomotor OR reflex OR risk-taking OR schizophrenia OR seizure* OR senil* OR sensation* OR sleep OR smell |

| **BIOSIS search terms** | | |
|---|---|---|
| | | OR sociab* OR spatial-behavior OR speech* OR spelling OR stereotypic-movement* OR stroke OR substantia-nigra OR synap* OR taste OR tauopath* OR Thyroglobulin OR Thyroid-disease* OR Thyroid-gland OR Thyroid-hormone* OR thyroiditis OR thyronine* OR thyrotoxicosis OR Thyrotropin OR thyroxine OR triiodothyronine OR vision OR visual-motor OR Visuospatial-processing OR Water-maze) |
| #3 | Final Search | #1 AND #2<br>Indexes=BCI, Timespan=All years<br><br>Refined by: RESEARCH AREAS: ( BIOCHEMISTRY MOLECULAR BIOLOGY OR NEUROSCIENCES NEUROLOGY OR ENDOCRINOLOGY METABOLISM OR PHARMACOLOGY PHARMACY OR TOXICOLOGY OR CELL BIOLOGY OR PHYSIOLOGY OR PUBLIC ENVIRONMENTAL OCCUPATIONAL HEALTH OR DENTISTRY ORAL SURGERY MEDICINE OR ENVIRONMENTAL SCIENCES ECOLOGY OR DEVELOPMENTAL BIOLOGY OR UROLOGY NEPHROLOGY OR PEDIATRICS OR LIFE SCIENCES BIOMEDICINE OTHER TOPICS OR GENETICS HEREDITY OR PSYCHIATRY OR REPRODUCTIVE BIOLOGY OR PATHOLOGY ) |

## *EMBASE*

**Date of search: 11/28/2016; 9,426 results**

| **EMBASE search terms** | | |
|---|---|---|
| #1 | Fluoride | ((Fluoride/exp OR fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis OR fluorosis/exp OR 'fluorosis, dental'/exp) NOT (f-labeled OR "fluorine-18" OR radioligand* OR 18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan))) |
| #2 | Neurological and Thyroid outcomes | ('Academic performance':ti,ab OR 'active-avoidance':ti,ab OR 'ADHD':ti,ab OR 'alzheimer*':ti,ab OR 'amygdala':ti,ab OR 'antisocial':ti,ab OR 'anxiety':ti,ab OR 'anxious':ti,ab OR 'asperger*':ti,ab OR 'attention deficit':ti,ab OR 'auditory':ti,ab OR 'autism':ti,ab OR 'autistic':ti,ab OR 'behavioral':ti,ab OR 'behaviors':ti,ab OR 'behavioural':ti,ab OR 'behaviours':ti,ab OR 'bipolar':ti,ab OR 'cerebellum':ti,ab OR 'cognition':ti,ab OR 'cognitive':ti,ab OR 'communication-disorder*':ti,ab OR 'comprehension':ti,ab OR 'cortical':ti,ab OR 'cranial':ti,ab OR 'delayed development':ti,ab OR 'dementia':ti,ab OR 'dendrit*':ti,ab OR 'dentate-gyrus':ti,ab OR 'depression':ti,ab OR 'developmental impairment':ti,ab OR 'developmental-delay*':ti,ab OR 'developmental-disorder*':ti,ab OR 'dextrothyroxine':ti,ab OR 'diiodothyronine*':ti,ab OR 'diiodotyrosine':ti,ab OR 'down syndrome':ti,ab OR 'dyslexia':ti,ab OR 'entorhinal cortex':ti,ab OR 'epilep*':ti,ab OR 'euthyroid':ti,ab OR 'gait':ti,ab OR 'gangli*':ti,ab OR 'glia*':ti,ab OR 'gliogenesis':ti,ab OR 'goiter':ti,ab OR 'graves-disease':ti,ab OR 'hearing':ti,ab OR 'hippocamp*':ti,ab OR 'human development':ti,ab OR 'hyperactiv*':ti,ab OR 'hyperthyroid*':ti,ab OR 'hypothalam*':ti,ab OR 'hypothyroid*':ti,ab OR 'impulse-control':ti,ab OR 'impulsiv*':ti,ab OR 'Intellectual disability':ti,ab OR 'intelligence':ti,ab OR 'iodide peroxidase':ti,ab OR 'IQ':ti,ab OR 'ischemi*':ti,ab OR 'language':ti,ab OR 'learning':ti,ab OR 'lewy bod*':ti,ab OR 'locomotor':ti,ab OR 'long-term potentiation':ti,ab OR 'long-term synaptic depression':ti,ab OR 'memory':ti,ab OR 'mental deficiency':ti,ab OR 'mental development':ti,ab OR 'mental disorder*':ti,ab OR 'mental illness':ti,ab OR 'mental recall':ti,ab OR 'mental-deficit':ti,ab OR 'mobility':ti,ab OR 'monoiodotyrosine':ti,ab OR 'mood':ti,ab OR 'morris-maze':ti,ab OR 'morris-water':ti,ab OR 'motor abilit*':ti,ab OR 'Motor activities':ti,ab OR 'Motor activity':ti,ab OR 'motor performance':ti,ab OR 'motor skill*':ti,ab OR 'multiple sclerosis':ti,ab OR 'myxedema':ti,ab OR 'nerve':ti,ab OR 'Nervous system':ti,ab OR 'nervous-system':ti,ab OR 'neural':ti,ab OR 'neurit*':ti,ab OR 'neurobehav*':ti,ab OR 'Neurocognitive impairment':ti,ab OR 'neurodegenerat*':ti,ab OR 'Neurodevelopment*':ti,ab OR 'neurodisease*':ti,ab OR 'neurologic*':ti,ab OR 'neuromuscular':ti,ab OR 'neuron*':ti,ab OR 'neuropath*':ti,ab OR 'obsessive compulsive':ti,ab OR 'OCD':ti,ab OR 'olfaction':ti,ab OR 'olfactory':ti,ab OR 'open-field-test':ti,ab OR 'optic':ti,ab OR 'palsy':ti,ab OR 'panic':ti,ab OR 'parahippocamp*':ti,ab OR 'paranoia':ti,ab OR 'paranoid':ti,ab OR 'parkinson*':ti,ab OR 'passive |

| **EMBASE search terms** | | |
|---|---|---|
| | | avoidance':ti,ab OR 'perception':ti,ab OR 'perforant*':ti,ab OR 'personality':ti,ab OR 'phobia':ti,ab OR 'plasticity':ti,ab OR 'problem solving':ti,ab OR 'proprioception':ti,ab OR 'psychomotor':ti,ab OR 'reflex':ti,ab OR 'risk taking':ti,ab OR 'schizophrenia':ti,ab OR 'seizure*':ti,ab OR 'senil*':ti,ab OR 'sensation*':ti,ab OR 'sleep':ti,ab OR 'smell':ti,ab OR 'sociab*':ti,ab OR 'spatial behavior':ti,ab OR 'speech*':ti,ab OR 'spelling':ti,ab OR 'stereotypic-movement*':ti,ab OR 'stroke':ti,ab OR 'substantia-nigra':ti,ab OR 'synap*':ti,ab OR 'taste':ti,ab OR 'tauopath*':ti,ab OR 'Thyroglobulin':ti,ab OR 'Thyroid disease*':ti,ab OR 'thyroid gland':ti,ab OR 'thyroid hormone*':ti,ab OR 'thyroiditis':ti,ab OR 'thyronine*':ti,ab OR 'thyrotoxicosis':ti,ab OR 'Thyrotropin':ti,ab OR 'thyroxine':ti,ab OR 'triiodothyronine':ti,ab OR 'vision':ti,ab OR 'visual motor':ti,ab OR 'Visuospatial processing':ti,ab OR 'water maze':ti,ab OR 'Alzheimer disease'/exp OR 'amygdala'/exp OR 'antisocial behavior'/exp OR 'anxiety'/exp OR 'Asperger syndrome'/exp OR 'attention deficit disorder'/exp OR 'autism'/exp OR 'behavior'/exp OR 'behavior disorder'/exp OR 'bipolar disorder'/exp OR 'cognition'/exp OR 'cognitive defect'/exp OR 'communication disorder'/exp OR 'communication disorders'/exp OR 'comprehension'/exp OR 'Constitutive androstane receptor'/exp OR 'dementia'/exp OR 'depression'/exp OR 'developmental delay'/exp OR 'developmental disorder'/exp OR 'dextrothyroxine'/exp OR 'diiodothyronine'/exp OR 'diiodotyrosine'/exp OR 'disorders of higher cerebral function'/exp OR 'disruptive behavior'/exp OR 'dissociative disorder'/exp OR 'dyslexia'/exp OR 'gait'/exp OR 'gait disorder'/exp OR 'Glucuronosyltransferase'/exp OR 'goiter'/exp OR 'graves-disease'/exp OR 'hearing'/exp OR 'high risk behavior'/exp OR 'hyperthyroidism'/exp OR 'impulse control disorder'/exp OR 'impulsiveness'/exp OR 'Intellectual disability'/exp OR 'intelligence'/exp OR 'intelligence quotient'/exp OR 'iodide peroxidase'/exp OR 'ischemia'/exp OR 'language'/exp OR 'learning'/exp OR 'locomotion'/exp OR 'Malate Dehydrogenase'/exp OR 'malate dehydrogenase (decarboxylating)'/exp OR 'memory'/exp OR 'mental deficiency'/exp OR 'mental development'/exp OR 'mental disease'/exp OR 'mood'/exp OR 'Motor activity'/exp OR 'motor dysfunction'/exp OR 'motor performance'/exp OR 'myxedema'/exp OR 'nerve cell'/exp OR 'nerve cell differentiation'/exp OR 'Nervous system'/exp OR 'neurobehavioral'/exp OR 'neurodegeneration'/exp OR 'Neurogranin'/exp OR 'neurologic disease'/exp OR 'neuromuscular disease'/exp OR 'neuropathic'/exp OR 'neuropathology'/exp OR 'neuropathy'/exp OR 'neurotox'/exp OR 'obsessive compulsive disorder'/exp OR 'olfactory system'/exp OR 'panic'/exp OR 'paralysis'/exp OR 'paranoia'/exp OR 'Parkinson disease'/exp OR 'patient mobility'/exp OR 'perception'/exp OR 'perception disorder'/exp OR 'personality'/exp OR 'phobia'/exp OR 'Pregnane X Receptor'/exp OR 'proprioception'/exp OR 'psychomotor activity'/exp OR 'psychomotor disorder'/exp OR 'recall'/exp OR 'thyroid hormone receptor'/exp OR 'thyrotropin receptor'/exp OR 'reflex'/exp OR 'Retinoid X Receptor'/exp OR 'seizure'/exp OR 'senile dementia'/exp OR 'senility'/exp OR |

| **EMBASE search terms** | | |
|---|---|---|
| | | 'sensation'/exp OR 'sleep'/exp OR 'social behavior'/exp OR 'speech'/exp OR 'Spelling'/exp OR 'taste'/exp OR 'tauopathy'/exp OR 'Thyroglobulin'/exp OR 'Thyroid disease'/exp OR 'thyroid diseases'/exp OR 'thyroid gland'/exp OR 'thyroid hormone'/exp OR 'thyroiditis'/exp OR 'thyronine'/exp OR 'thyrotoxicosis'/exp OR 'Thyrotropin'/exp OR 'thyroxine'/exp OR 'vision'/exp OR 'visuomotor coordination'/exp) |
| #3 | Final Search | #1 AND #2<br>Embase OR Embase Classic |

***PsycINFO***

**Date of search: 11/28/2016; 181 results**

| **PsycINFO search terms** | | |
|---|---|---|
| #1 | Fluoride | **Title OR Abstract**: ((fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) NOT (f-labeled OR "fluorine-18" OR radioligand* OR 18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan))) |
| #2 | Neurological and Thyroid outcomes | **Title OR Abstract:** (Academic-performance OR active-avoidance OR ADHD OR alzheimer* OR amygdala OR antisocial OR anxiety OR anxious OR asperger* OR attention-deficit OR auditory OR autism OR autistic OR behavioral OR behaviors OR behavioural OR behaviours OR bipolar OR cerebellum OR cognition OR cognitive OR communication-disorder* OR comprehension OR cortical OR cranial OR delayed-development OR dementia OR dendrit* OR dentate-gyrus OR depression OR developmental-impairment OR Developmental-delay* OR developmental-disorder* OR dextrothyroxine OR diiodothyronine* OR diiodotyrosine OR down-syndrome OR dyslexia OR entorhinal-cortex OR epilep* OR euthyroid OR gait OR gangli* OR glia* OR gliogenesis OR goiter OR graves-disease OR hearing OR hippocamp* OR human-development OR hyperactiv* OR hyperthyroid* OR hypothalam* OR hypothyroid* OR impulse-control OR impulsiv* OR Intellectual-disability OR intelligence OR iodide-peroxidase OR IQ OR ischemi* OR language OR learning OR lewy-bod* OR locomotor OR long-term-potentiation OR long-term-synaptic-depression OR memory OR mental-deficiency OR mental-development OR mental-disorder* OR mental-illness OR mental-recall OR mental-deficit OR mobility OR monoiodotyrosine OR mood OR morris-maze OR morris-water OR motor-abilit* OR Motor-activities OR Motor-activity OR Motor-performance OR Motor-skill* OR Multiple-sclerosis OR myxedema OR nerve OR Nervous-system OR neural OR neurit* OR neurobehav* OR Neurocognitive-impairment OR neurodegenerat* OR Neurodevelopment* OR neurodisease* OR neurologic* OR neuromuscular OR neuron* OR neuropath* OR obsessive-compulsive OR OCD OR olfaction OR olfactory OR open-field-test OR optic OR palsy OR panic OR parahippocamp* OR paranoia OR paranoid OR parkinson* OR passive-avoidance OR perception OR perforant* OR personality OR phobia OR plasticity OR problem-solving |

| PsycINFO search terms | | |
|---|---|---|
| | | OR proprioception OR psychomotor OR reflex OR risk-taking OR schizophrenia OR seizure* OR senil* OR sensation* OR sleep OR smell OR sociab* OR spatial-behavior OR speech* OR spelling OR stereotypic-movement* OR stroke OR substantia-nigra OR synap* OR taste OR tauopath* OR Thyroglobulin OR Thyroid-disease* OR Thyroid-gland OR Thyroid-hormone* OR thyroiditis OR thyronine* OR thyrotoxicosis OR Thyrotropin OR thyroxine OR triiodothyronine OR vision OR visual-motor OR Visuospatial-processing OR Water-maze) |
| #3 | Final Search | #1 OR #2 |

### *PubMed*

**Date of search: 12/19/2016; 7,264 results**

| PubMed search terms | | |
|---|---|---|
| #1 | Fluoride | ((Fluorides[mh:noexp] OR fluorides, topical[mh] OR sodium fluoride[mh] OR Fluorosis, Dental[mh] OR fluorosis[tiab] OR fluorid*[tiab] OR flurid*[tiab] OR fluorin*[tiab] OR florin*[tiab]) NOT (18F[tiab] OR f-18[tiab] OR 19F[tiab] OR f-19[tiab] OR f-labeled[tiab] OR "fluorine-18"[tiab] OR "fluorine-19"[tiab] OR pet-scan[tiab] OR radioligand*[tiab])) |
| #2 | Neurological and Thyroid outcomes | AND ((Aryl Hydrocarbon Hydroxylases[mh] OR Aryl Hydrocarbon Receptor Nuclear Translocator[mh] OR Behavior and Behavior Mechanisms[mh] OR Gene Expression Regulation[mh] OR Glucuronosyltransferase[mh] OR Intelligence tests[mh] OR Malate Dehydrogenase[mh] OR Mediator Complex Subunit 1[mh] OR Mental disorders[mh] OR Mental processes[mh] OR Monocarboxylic Acid Transporters[mh] OR Myelin Basic Protein[mh] OR nervous system[mh] OR nervous system diseases[mh] OR nervous system physiological phenomena[mh] OR Neurogranin[mh] OR Oligodendroglia[mh] OR Peroxisome Proliferator-Activated Receptors[mh] OR Psychological Phenomena and Processes[mh] OR Receptors, thyroid hormone[mh] OR Receptors, thyrotropin[mh] OR Retinoid X Receptors[mh] OR thyroid diseases[mh] OR thyroid hormones[mh] OR Thyrotropin-releasing hormone[mh] OR Thyroxine-Binding Proteins[mh] OR Pregnane X Receptor[supplementary concept] OR thyroid-hormone-receptor interacting protein[supplementary concept] OR Constitutive androstane receptor[supplementary concept] OR Academic performance[tiab] OR auditory[tiab] OR cortical[tiab] OR delayed development[tiab] OR developmental impairment[tiab] OR developmental-delay*[tiab] OR developmental-disorder*[tiab] OR euthyroid[tiab] OR gait[tiab] OR glia*[tiab] OR gliogenesis[tiab] OR hyperactiv*[tiab] OR impulse-control[tiab] OR iodide peroxidase[tiab] OR IQ[tiab] OR ischemi*[tiab] OR locomotor[tiab] OR mental deficiency[tiab] OR mental development[tiab] OR mental illness[tiab] OR mental-deficit[tiab] OR mobility[tiab] OR mood[tiab] OR morris-maze[tiab] OR morris-water[tiab] OR motor abilit*[tiab] OR Motor |

| | | |
|---|---|---|
| | | activities[tiab] OR motor performance[tiab] OR nerve[tiab] OR neural[tiab] OR neurobehav*[tiab] OR Neurocognitive impairment[tiab] OR neurodegenerat*[tiab] OR Neurodevelopment*[tiab] OR neurodisease*[tiab] OR neurologic*[tiab] OR neuromuscular[tiab] OR neuron*[tiab] OR neuropath*[tiab] OR obsessive compulsive[tiab] OR OCD[tiab] OR olfaction[tiab] OR olfactory[tiab] OR open-field-test[tiab] OR passive avoidance[tiab] OR plasticity[tiab] OR senil*[tiab] OR sociab*[tiab] OR speech*[tiab] OR spelling[tiab] OR stereotypic-movement*[tiab] OR synap*[tiab] OR tauopath*[tiab] OR Thyroglobulin[tiab] OR Thyroid disease*[tiab] OR thyroid gland[tiab] OR thyroid hormone*[tiab] OR thyronine*[tiab] OR visual motor[tiab] OR Visuospatial processing[tiab] OR water maze[tiab]) OR ((active-avoidance[tiab] OR ADHD[tiab] OR alzheimer*[tiab] OR amygdala[tiab] OR antisocial[tiab] OR anxiety[tiab] OR anxious[tiab] OR asperger*[tiab] OR attention deficit[tiab] OR autism[tiab] OR autistic[tiab] OR behavioral[tiab] OR behaviors[tiab] OR behavioural[tiab] OR behaviours[tiab] OR bipolar[tiab] OR cerebellum[tiab] OR cognition[tiab] OR cognitive[tiab] OR communication-disorder*[tiab] OR comprehension[tiab] OR cranial[tiab] OR dementia[tiab] OR dendrit*[tiab] OR dentate-gyrus[tiab] OR depression[tiab] OR dextrothyroxine[tiab] OR diiodothyronine*[tiab] OR diiodotyrosine[tiab] OR down syndrome[tiab] OR dyslexia[tiab] OR entorhinal cortex[tiab] OR epilep*[tiab] OR gangli*[tiab] OR goiter[tiab] OR graves-disease[tiab] OR hearing[tiab] OR hippocamp*[tiab] OR human development[tiab] OR hyperthyroid*[tiab] OR hypothalam*[tiab] OR hypothyroid*[tiab] OR impulsiv*[tiab] OR Intellectual disability[tiab] OR intelligence[tiab] OR language[tiab] OR learning[tiab] OR lewy bod*[tiab] OR long-term potentiation[tiab] OR long-term synaptic depression[tiab] OR memory[tiab] OR mental disorder*[tiab] OR mental recall[tiab] OR monoiodotyrosine[tiab] OR Motor activity[tiab] OR motor skill*[tiab] OR multiple sclerosis[tiab] OR myxedema[tiab] OR Nervous system[tiab] OR nervous-system[tiab] OR neurit*[tiab] OR optic[tiab] OR palsy[tiab] OR panic[tiab] OR parahippocamp*[tiab] OR paranoia[tiab] OR paranoid[tiab] OR parkinson*[tiab] OR perception[tiab] OR perforant*[tiab] OR personality[tiab] OR phobia[tiab] OR problem solving[tiab] OR proprioception[tiab] OR psychomotor[tiab] OR reflex[tiab] OR risk taking[tiab] OR schizophrenia[tiab] OR seizure*[tiab] OR sensation*[tiab] OR sleep[tiab] OR smell[tiab] OR spatial behavior[tiab] OR stroke[tiab] OR substantia-nigra[tiab] OR taste[tiab] OR thyroiditis[tiab] OR thyrotoxicosis[tiab] OR Thyrotropin[tiab] OR thyroxine[tiab] OR triiodothyronine[tiab] OR vision[tiab]) NOT medline[sb])) |
| #3 | Final Search | #1 AND #2 |

### *Web of Science*

**Date of search: 11/28/2016; 3,336 results**

| **Web of Science search terms** | | |
|---|---|---|
| #1 | Fluoride | **TOPIC:** ((fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) NOT (f-labeled OR "fluorine-18" OR radioligand* OR 18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan))) |
| #2 | Neurological and Thyroid outcomes | **TOPIC:** (Academic-performance OR active-avoidance OR ADHD OR alzheimer* OR amygdala OR antisocial OR anxiety OR anxious OR asperger* OR attention-deficit OR auditory OR autism OR autistic OR behavioral OR behaviors OR behavioural OR behaviours OR bipolar OR cerebellum OR cognition OR cognitive OR communication-disorder* OR comprehension OR cortical OR cranial OR delayed-development OR dementia OR dendrit* OR dentate-gyrus OR depression OR developmental-impairment OR Developmental-delay* OR developmental-disorder* OR dextrothyroxine OR diiodothyronine* OR diiodotyrosine OR down-syndrome OR dyslexia OR entorhinal-cortex OR epilep* OR euthyroid OR gait OR gangli* OR glia* OR gliogenesis OR goiter OR graves-disease OR hearing OR hippocamp* OR human-development OR hyperactiv* OR hyperthyroid* OR hypothalam* OR hypothyroid* OR impulse-control OR impulsiv* OR Intellectual-disability OR intelligence OR iodide-peroxidase OR IQ OR ischemi* OR language OR learning OR lewy-bod* OR locomotor OR long-term-potentiation OR long-term-synaptic-depression OR memory OR mental-deficiency OR mental-development OR mental-disorder* OR mental-illness OR mental-recall OR mental-deficit OR mobility OR monoiodotyrosine OR mood OR morris-maze OR morris-water OR motor-abilit* OR Motor-activities OR Motor-activity OR Motor-performance OR Motor-skill* OR Multiple-sclerosis OR myxedema OR nerve OR Nervous-system OR neural OR neurit* OR neurobehav* OR Neurocognitive-impairment OR neurodegenerat* OR Neurodevelopment* OR neurodisease* OR neurologic* OR neuromuscular OR neuron* OR neuropath* OR obsessive-compulsive OR OCD OR olfaction OR olfactory OR open-field-test OR optic OR palsy OR panic OR parahippocamp* OR paranoia OR paranoid OR parkinson* OR passive-avoidance OR perception OR perforant* OR personality OR phobia OR plasticity OR problem-solving OR proprioception OR psychomotor OR reflex OR risk-taking OR schizophrenia OR seizure* OR senil* OR sensation* OR sleep OR smell OR sociab* OR spatial-behavior OR speech* OR spelling OR stereotypic-movement* OR stroke OR substantia-nigra OR synap* OR taste OR tauopath* OR Thyroglobulin OR Thyroid-disease* OR |

| **Web of Science search terms** | | |
|---|---|---|
| | | Thyroid-gland OR Thyroid-hormone* OR thyroiditis OR thyronine* OR thyrotoxicosis OR Thyrotropin OR thyroxine OR triiodothyronine OR vision OR visual-motor OR Visuospatial-processing OR Water-maze) |
| #3 | Final Search | #1 AND #2<br>Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED, IC Timespan=All years<br>Refined by: RESEARCH AREAS: (DEVELOPMENTAL BIOLOGY OR RESPIRATORY SYSTEM OR DENTISTRY ORAL SURGERY MEDICINE OR BIOCHEMISTRY MOLECULAR BIOLOGY OR PHARMACOLOGY PHARMACY OR LIFE SCIENCES BIOMEDICINE OTHER TOPICS OR ENVIRONMENTAL SCIENCES ECOLOGY OR TOXICOLOGY OR PUBLIC ENVIRONMENTAL OCCUPATIONAL HEALTH OR PSYCHIATRY OR PATHOLOGY OR NEUROSCIENCES NEUROLOGY OR BEHAVIORAL SCIENCES OR VETERINARY SCIENCES OR NUTRITION DIETETICS OR ENDOCRINOLOGY METABOLISM OR PSYCHOLOGY OR MARINE FRESHWATER BIOLOGY OR CELL BIOLOGY OR PHYSIOLOGY OR REPRODUCTIVE BIOLOGY OR PEDIATRICS) |

***SCOPUS***

**Date of search: 11/28/2016; 5,222 results**

| **SCOPUS search terms** | | |
|---|---|---|
| #1 | Fluoride | **Title OR Abstract**: ((fluorid* OR flurid* OR fluorin* OR florin* OR fluorosis) AND NOT (f-labeled OR "fluorine-18" OR radioligand* OR 18F OR F-18 OR "fluorine-18" OR 19F OR F-19 OR "fluorine-19" OR (PET AND scan))) |
| #2 | Neurological and Thyroid outcomes | **Title OR Abstract:** (Academic-performance OR active-avoidance OR ADHD OR alzheimer* OR amygdala OR antisocial OR anxiety OR anxious OR asperger* OR attention-deficit OR auditory OR autism OR autistic OR behavioral OR behaviors OR behavioural OR behaviours OR bipolar OR cerebellum OR cognition OR cognitive OR communication-disorder* OR comprehension OR cortical OR cranial OR delayed-development OR dementia OR dendrit* OR dentate-gyrus OR depression OR developmental-impairment OR Developmental-delay* OR developmental-disorder* OR dextrothyroxine OR diiodothyronine* OR diiodotyrosine OR down-syndrome OR dyslexia OR entorhinal-cortex OR epilep* OR euthyroid OR gait OR gangli* OR glia* OR gliogenesis OR goiter OR graves-disease OR hearing OR hippocamp* OR human-development OR hyperactiv* OR hyperthyroid* OR hypothalam* OR hypothyroid* OR impulse-control OR impulsiv* OR Intellectual-disability OR intelligence OR iodide-peroxidase OR IQ OR |

| SCOPUS search terms | | |
|---|---|---|
| | | ischemi* OR language OR learning OR lewy-bod* OR locomotor OR long-term-potentiation OR long-term-synaptic-depression OR memory OR mental-deficiency OR mental-development OR mental-disorder* OR mental-illness OR mental-recall OR mental-deficit OR mobility OR monoiodotyrosine OR mood OR morris-maze OR morris-water OR motor-abilit* OR Motor-activities OR Motor-activity OR Motor-performance OR Motor-skill* OR Multiple-sclerosis OR myxedema OR nerve OR Nervous-system OR neural OR neurit* OR neurobehav* OR Neurocognitive-impairment OR neurodegenerat* OR Neurodevelopment* OR neurodisease* OR neurologic* OR neuromuscular OR neuron* OR neuropath* OR obsessive-compulsive OR OCD OR olfaction OR olfactory OR open-field-test OR optic OR palsy OR panic OR parahippocamp* OR paranoia OR paranoid OR parkinson* OR passive-avoidance OR perception OR perforant* OR personality OR phobia OR plasticity OR problem-solving OR proprioception OR psychomotor OR reflex OR risk-taking OR schizophrenia OR seizure* OR senil* OR sensation* OR sleep OR smell OR sociab* OR spatial-behavior OR speech* OR spelling OR stereotypic-movement* OR stroke OR substantia-nigra OR synap* OR taste OR tauopath* OR Thyroglobulin OR Thyroid-disease* OR Thyroid-gland OR Thyroid-hormone* OR thyroiditis OR thyronine* OR thyrotoxicosis OR Thyrotropin OR thyroxine OR triiodothyronine OR vision OR visual-motor OR Visuospatial-processing OR Water-maze) |
| #3 | Final Search | #1 AND #2<br>AND ( LIMIT-TO(SUBJAREA,"MEDI" ) OR LIMIT-TO(SUBJAREA,"BIOC" ) OR LIMIT-TO(SUBJAREA,"ENVI" ) OR LIMIT-TO(SUBJAREA,"PHAR" ) OR LIMIT-TO(SUBJAREA,"AGRI" ) OR LIMIT-TO(SUBJAREA,"NEUR" ) OR LIMIT-TO(SUBJAREA,"MULT" ) OR LIMIT-TO(SUBJAREA,"PSYC" ) OR LIMIT-TO(SUBJAREA,"Undefined" ) ) |

# Appendix 2. DATA EXTRACTION ELEMENTS FOR HAWC: HUMAN STUDIES

| Data extraction elements for human studies | |
|---|---|
| ***Funding*** | Funding source(s) |
| | Reporting of COI by authors and/or translators (*reporting bias) |
| ***Subjects*** | Study population name/description |
| | Dates of study and sampling time frame |
| | Geography (country, region, state, etc.) |
| | Demographics (sex, race/ethnicity, age or lifestage and exposure and outcome assessment) |
| | Number of subjects (target, enrolled, n per group in analysis, and participation/follow-up rates) (*missing data bias) |

| **Data extraction elements for human studies** | |
|---|---|
| | Inclusion/exclusion criteria/recruitment strategy (*selection bias) |
| | Description of reference group (*selection bias) |
| ***Methods*** | Study design (e.g., prospective or retrospective cohort, nested case-control study, cross-sectional, population-based case-control study, intervention, case report, etc.) |
| | Length of follow-up (*information bias) |
| | Health outcome category, e.g., neurodevelopment |
| | Health outcome, e.g., memory (*reporting bias) |
| | Diagnostic or methods used to measure health outcome (*information bias) |
| | Confounders or modifying factors and how considered in analysis (e.g., included in final model, considered for inclusion but determined not needed (*confounding bias) |
| | Substance name and CAS number |
| | Exposure assessment (e.g., blood, urine, hair, air, drinking water, job classification, residence, administered treatment in controlled study, etc.) (*information bias) |
| | Methodological details for exposure assessment (e.g., HPLC-MS/MS, limit of detection) (*information bias) |
| | Statistical methods (*information bias) |
| ***Results*** | Exposure levels (e.g., mean, median, measures of variance as presented in paper, such as SD, SEM, 75th/90th/95th percentile, minimum/maximum); range of exposure levels, number of exposed cases |
| | Statistical findings (e.g., adjusted β, standardized mean difference, adjusted odds ratio, standardized mortality ratio, relative risk, etc.) or description of qualitative results. When possible, OHAT will convert measures of effect to a common metric with associated 95% confidence intervals (CI). Most often, measures of effect for continuous data are expressed as mean difference, standardized mean difference, and percent control response. Categorical data are typically expressed as odds ratio, relative risk (RR, also called risk ratio), or β values, depending on what metric is most commonly reported in the included studies and on OHAT's ability to obtain information for effect conversions from the study or through author query. |

| Data extraction elements for human studies | |
|---|---|
| | If not presented in the study, statistical power can be assessed during data extraction using an approach that can detect a 10% to 20% change from response by control or referent group for continuous data, or a relative risk or odds ratio of 1.5 to 2 for categorical data, using the prevalence of exposure or prevalence of outcome in the control or referent group to determine sample size. For categorical data where the sample sizes of exposed and control or referent groups differ, the sample size of the exposed group will be used to determine the relative power category. Recommended sample sizes to achieve 80% power for a given effect size, i.e., 10% or 20% change from control, will be compared to sample sizes used in the study to categorize statistical power as "appears to be adequately powered" (sample size for 80% power met), somewhat underpowered (sample size is 75% to < 100% of number required for 80% power), "underpowered" (sample size is 50% to < 75% of number required for 80% power), or "severely underpowered" (sample size is < 50% of number required for 80% power). |
| | Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, non-monotonic) |
| ***Other*** | Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, and statistical result conversions, etc. |

# Appendix 3.DATA EXTRACTION ELEMENTS FOR HAWC: ANIMAL STUDIES

| Data extraction elements for animal studies | |
|---|---|
| ***Funding*** | Funding source(s) |
| | Reporting of COI by authors and/or translators (*reporting bias) |
| ***Animal Model*** | Sex |
| | Species |
| | Strain |
| ***Treatment*** | Chemical name and CAS number |
| | Source of chemical |
| | Purity of chemical (*information bias) |
| | Dose levels or concentration (as presented and converted to mg/kg bw/d when possible) |
| | Other dose-related details, such as whether administered dose level was verified by measurement, information on internal dosimetry (*information bias) |
| | Vehicle used for exposed animals |
| | Route of administration (e.g., oral, inhalation, dermal, injection) |
| | Age or lifestage at start of dosing and at health outcome assessment |

| **Data extraction elements for animal studies** | |
|---|---|
| | Duration and frequency of dosing (e.g., hours, days, weeks when administration was ended, days per week) |
| ***Methods*** | Study design (e.g., single treatment, acute, subchronic (e.g., 90 days in a rodent), chronic, multigenerational, developmental, other) |
| | Guideline compliance (i.e., use of EPA, OECD, NTP or another guideline for study design, conducted under GLP guideline conditions, non-GLP but consistent with guideline study, non-guideline peer-reviewed publication) |
| | Number of animals per group (and dams per group in developmental studies) (*missing data bias) |
| | Randomization procedure, allocation concealment, blinding during outcome assessment (*selection bias) |
| | Method to control for litter effects in developmental studies (*information bias) |
| | Use of negative controls and whether controls were untreated, vehicle-treated, or both |
| | Endpoint health category (e.g., reproductive) |
| | Endpoint (e.g., infertility) |
| | Diagnostic or method to measure endpoint (*information bias) |
| | Statistical methods (*information bias) |
| ***Results*** | Measures of effect at each dose or concentration level (e.g., mean, median, frequency, measures of precision or variance) or description of qualitative results. When possible, OHAT will convert measures of effect to a common metric with associated 95% confidence intervals (CI). Most often, measures of effect for continuous data will be expressed as percent control response, mean difference, or standardized mean difference. Categorical data will be expressed as relative risk (RR, also called risk ratio). |
| | No observed effect level (NOEL), lowest observed effect level (LOEL), benchmark dose (BMD) analysis, statistical significance of other dose levels, or other estimates of effect presented in paper. **Note:** The NOEL and LOEL are highly influenced by study design, give no quantitative information about the relationship between dose and response, and can be subject to author's interpretation (e.g., a statistically significant effect might not be considered biologically important). Also, a NOEL does not necessarily mean zero response. Ideally, the response rate or effect size at specific dose levels is used as the primary measure to characterize the response. |

| **Data extraction elements for animal studies** | |
|---|---|
| | If not presented in the study, statistical power can be assessed during data extraction using an approach that assesses the ability to detect a 10% to 20% change from control group's response for continuous data, or a relative risk or odds ratio of 1.5–2 for categorical data, using the outcome frequency in the control group to determine sample size. Recommended sample sizes to achieve 80% power for a given effect size, i.e., 10% or 20% change from control, will be compared to sample sizes used in the study to categorize statistical power. Studies will be considered adequately powered when sample size for 80% power is met. |
| | Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, nonmonotonic) |
| | Data on internal concentration, toxicokinetics, or toxicodynamics (when reported) |
| ***Other*** | Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, statistical result conversions, etc. |

# Appendix 4.DATA EXTRACTION ELEMENTS FOR HAWC: IN VITRO STUDIES

| **Data extraction elements for in vitro studies** | |
|---|---|
| ***Funding*** | Funding source(s) |
| | Reporting of COI by authors and/or translators (*reporting bias) |
| ***Cell/Tissue Model*** | Cell line, cell type, or tissue |
| | Source of cells/tissues (and validation of identity) |
| | Sex of human/animal origin |
| | Species |
| | Strain |
| ***Treatment*** | Chemical name and CAS number |
| | Concentration levels (as presented and converted to µM when possible) |
| | Source of chemical |
| | Purity of chemical (*information bias) |
| | Vehicle used for experimental/control conditions |
| | Duration and frequency of dosing (e.g., hours, days, weeks when administration was ended, days per week) |
| ***Methods*** | Guideline compliance (i.e., use of EPA, OECD, NTP or another guideline for study design, conducted under GLP guideline conditions, non-GLP but consistent with guideline study, non-guideline peer-reviewed publication) |
| | Randomization procedure, allocation concealment, blinding during outcome assessment (*selection bias) |
| | Number of replicates per group (*information bias) |
| | Percent serum/plasma in medium |
| | Use of negative controls and whether controls were untreated, vehicle-treated, or both |
| | Report on data from positive controls – was expected response observed? (*information bias) |
| | Endpoint health category (e.g. neurological and thyroid) |
| | Endpoint or assay target (e.g., T3, T4, TSH levels). |
| | Name and source of assay kit |
| | Diagnostic or method to measure endpoint (e.g., reporter gene)(*information bias) |
| | Statistical methods (*information bias) |

| **Data extraction elements for in vitro studies** | |
|---|---|
| ***Results*** | Measures of effect at each dose or concentration level (e.g., mean, median, frequency, and measures of precision or variance) or description of qualitative results. When possible, OHAT will convert measures of effect to a common metric with associated 95% confidence intervals (CI). Most often, measures of effect for continuous data will be expressed as mean difference, standardized mean difference, and percent control response. Categorical data will be expressed as relative risk (RR, also called risk ratio). |
| | No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC), statistical significance of other concentration levels, AC50, or other estimates of effect presented in paper. Note: The NOEC and LOEC are highly influenced by study design, do not give any quantitative information about the relationship between dose and response, and can be subject to author's interpretation (e.g., a statistically significant effect may not be considered biologically important). Also, a NOEC does not necessarily mean zero response. |
| | Observations on dose response (e.g., trend analysis, description of whether dose-response shape appears to be monotonic, non-monotonic) |
| ***Other*** | Documentation of author queries, use of digital rulers to estimate data values from figures, exposure unit, statistical result conversions, etc. |

# Appendix 5. RISK-OF-BIAS CRITERIA

The OHAT risk-of-bias tool for human and animal studies (version date January 2015 and available at https://ntp.niehs.nih.gov/go/38673) reflects OHAT's current best practices and provides the detailed discussion and instructions for the risk-of-bias practices used in this evaluation. The OHAT tool uses a single set of questions (also called "elements" or "domains") to assess risk-of-bias across various study types to facilitate consideration of conceptually similar potential sources of bias across the human and animal evidence streams with a common terminology. Individual risk-of-bias questions are designated as only applicable to certain study designs (e.g., cohort studies or experimental animal studies), and a subset of the questions apply to each study design (**Table 3**).

The specific criteria used to assess risk-of-bias for this evaluation are outlined below for human/observational studies and experimental animal studies. Based on literature searches, we do not expect any controlled exposure studies in humans (i.e., human controlled trials) or case-control studies and therefore have not included risk-of-bias criteria for these study designs. If relevant human controlled trials of fluoride are identified, the criteria from the January 2015 OHAT risk–of-bias tool will be used to evaluate risk-of-bias.

## *Observational Studies (Human studies)*

**Cross Sectional Studies**

***1. Was administered dose or exposure level adequately randomized? [NA]***

***2. Was allocation to study groups adequately concealed? [NA]***

***3. Did selection of study participants result in the appropriate comparison groups? [NA to Case series]***

| **Q3 Cross-sectional - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that subjects (both exposed and non-exposed) were similar (e.g., recruited from the same eligible population, recruited using the same inclusion and exclusion criteria, and were of similar age, socioeconomic, and health status), recruited within the same time frame, and had similar participation/response rates.<br>**Note:** A study will generally be considered low risk-of-bias if baseline characteristics of groups differed but these differences were considered as potential confounding or stratification variables (see question #4). |

| **Q3 Cross-sectional - Probably Low Risk-of-bias (+)** |
|---|
| Indirect evidence that subjects (both exposed and non-exposed) were similar (e.g., recruited from the same eligible population, recruited using the same inclusion and exclusion criteria, and were of similar age and health status) recruited within the same time frame, and had similar participation/response rates,<br>**OR** there is indirect evidence that differences between groups were not likely to substantively bias results.<br>**Note:** Includes studies where the authors state that characteristics of exposed and referent groups were similar (as above), but do not provide quantitative information on covariates. |

**Q3 Cross-sectional - Probably High Risk-of-bias (-) or (NR)**

Indirect evidence that subjects (both exposed and non-exposed) were not similar, recruited within very different time frames, or had the very different participation/response rates,

**OR** there is insufficient information provided about the comparison group to determine similarity to exposed groups (record "NR" as basis for answer).

**Q3 Cross-sectional - Definitely High Risk-of-bias (--)**

Direct evidence that subjects (both exposed and non-exposed) were not similar (e.g., recruited from the different eligible populations, recruited using different inclusion and exclusion criteria, or were significantly different in terms of age, socioeconomic, or health status), recruited within very different time frames, or had the very different participation/response rates.

***4. Did study design or analysis account for important confounding and modifying variables?***

**Q4 Cross-sectional - Definitely Low Risk-of-bias (++)**

Direct evidence that appropriate adjustments or explicit considerations were made for the variables listed below as potential confounders and/or effect measure modifiers in the final analyses through the use of statistical models to reduce research-specific bias including standardization, matching, adjustment in multivariate model, stratification, or other methods that were appropriately justified. Acceptable consideration of appropriate adjustment factors includes cases when the factor is not included in the final adjustment model because the author conducted analyses that indicated it did not need to be included,

**AND** there is direct evidence that primary covariates and confounders (including known neurodevelopmental toxicants lead and arsenic) were appropriately measured (using valid and reliable methods) and adjusted for,

**OR** there is direct evidence that certain covariates and cofounders that are anticipated to bias results were not present.

**Note:** The following variables should be considered as potential confounders and/or effect measure modifiers for the relationship between fluoride exposure and neurobehavioral outcomes**:** age, child's sex, race/ethnicity, maternal demographics (e.g., maternal age, BMI), parental behavioral and mental health disorders (e.g., ADHD, depression), socioeconomic status (e.g., maternal education, household income, marital status, crowding), smoking (e.g., maternal smoking status, secondhand tobacco smoke exposure), reproductive factors (e.g., parity), nutrition (e.g., BMI, growth, anemia), iodine deficiency/excess, minerals and other chemicals in water associated with neurotoxicity (e.g., arsenic, and lead), maternal (and paternal) IQ, quantity and quality of caregiving environment (e.g., HOME score).

**Note:** Many studies report incidence of dental and/or skeletal fluorosis, and sometimes stratify results by fluorosis severity. Because fluorosis is highly correlated with fluoride exposure, one should consider how the fluorosis is handled in the study, especially if the study authors adjusted for fluorosis.

**Q4 Cross-sectional - Probably Low Risk-of-bias (+)**

Indirect evidence that appropriate adjustments were made,

**AND** there is indirect evidence that potential covariates and confounders [age, child's sex, race/ethnicity, maternal demographics (e.g., maternal age, mother's cohort, BMI), parental behavioral and mental health disorders (e.g., ADHD, depression), socioeconomic status (e.g., maternal education, household income, marital status, crowding), smoking (e.g., maternal smoking status, secondhand tobacco smoke exposure), reproductive factors (e.g., parity),

| **Q4 Cross-sectional - Probably Low Risk-of-bias (+)** |
|---|
| nutrition (e.g., BMI, growth, anemia), iodine deficiency/excess, mineral and other chemicals in water associated with neurotoxicity (e.g., arsenic and lead), maternal (and paternal) IQ, quantity and quality of caregiving environment (e.g., HOME score)] were appropriately measured and adjusted for,<br>**OR** it is deemed that not considering or only considering a partial list of covariates or confounders in the final analyses would not appreciably bias results,<br>**AND** there is evidence (direct or indirect) that covariates and confounders considered were assessed using valid and reliable measurements,<br>**OR** it is deemed that the measures used would not appreciably bias results (i.e., the authors justified the validity of the measures from previously published research),<br>**AND** there is evidence (direct or indirect) that other co-exposures anticipated to bias results were not present or were appropriately adjusted for,<br>**OR** it is deemed that co-exposures present would not appreciably bias results. |

| **Q4 Cross-sectional - Probably High Risk-of-bias (-) or (NR)** |
|---|
| Indirect evidence that the distribution of important covariates and known confounders differed between the groups and was not appropriately adjusted for in the final analyses,<br>**OR** there is insufficient information provided about the distribution of known confounders (record "NR" as basis for answer),<br>**OR** there is indirect evidence that there was an unbalanced distribution of co-exposures that could affect neurological development across the primary study groups, which were not appropriately adjusted for,<br>**OR** there is indirect evidence that covariates and confounders considered were assessed using measurements of unknown validity,<br>**OR** there is insufficient information provided about the measurement techniques used to assess covariates and confounders considered (record "NR" as basis for answer). |

| **Q4 Cross-sectional - Definitely High Risk-of-bias (--)** |
|---|
| Direct evidence that the distribution of important covariates, known confounders, and co-exposures differed between the groups and was not accounted for,<br>**OR** confounding was demonstrated or likely to be present but not appropriately adjusted for in the final analyses,<br>**OR** there is direct evidence that covariates and confounders considered were assessed using non valid measurements,<br>**OR** there is indirect evidence of co-exposure to high levels of lead and arsenic (or other agents associated with negative effects on cognition) but these co-exposures were not appropriately measured and adjusted for. |

***5. Were experimental conditions identical across study groups? [NA]***

***6. Were the research personnel blinded to the study group during the study? [NA]***

***7. Were outcome data complete without attrition or exclusion from analysis?***

| **Q7 Cross-sectional - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that exclusion of subjects from analyses was adequately addressed, and reasons were documented when subjects were removed from the study or excluded from analyses. |

| Q7 Cross-sectional - Probably Low Risk-of-bias (+) |
|---|
| Indirect evidence that exclusion of subjects from analyses was adequately addressed, and reasons were documented when subjects were removed from the study or excluded from analyses. |

| Q7 Cross-sectional - Probably High Risk-of-bias (-) or (NR) |
|---|
| Indirect evidence that exclusion of subjects from analyses was not adequately addressed,<br>**OR** there is insufficient information provided about why subjects were removed from the study or excluded from analyses (record "NR" as basis for answer). |

| Q7 Cross-sectional - Definitely High Risk-of-bias (--) |
|---|
| Direct evidence that exclusion of subjects from analyses was not adequately addressed.<br>**Note**: Unacceptable handling of subject exclusion from analyses includes: reason for exclusion likely to be related to true outcome, with either imbalance in numbers or reasons for exclusion across study groups. |

***8. Can we be confident in the exposure/intake characterization?***

| Q8 Cross-sectional - Definitely Low Risk-of-bias (++) |
|---|
| Direct evidence that exposure or intake was consistently assessed (i.e., using the same method and during the same time-frame) using well-established methods that directly characterize exposure or intake,<br>**OR** fluoride intake is estimated from exposure sources, such as drinking water, that are well-characterized,<br>**OR** exposure was assessed using less-established methods that directly measure exposure and are validated against well-established methods,<br>**AND** there is sufficient range or variation in exposure measurements across groups to potentially identify associations with health outcomes,<br>**AND** there is evidence that most of the exposure data measurements are above the limit of quantitation for the assay such that different exposure groups can be distinguished,<br>**AND** there is evidence (direct or indirect) that the study used appropriate quality control (including blanks and spiked samples) or all analytical methods.<br>**Note:** Includes studies that measure fluoride in subjects' household drinking water or urine because the relationship between levels of fluoride in drinking water and urinary fluoride levels is generally well-characterized (ATSDR, 2003).<br>**Note:** Includes studies with direct intake estimates (e.g., studies that use high-quality measurements and validated estimation techniques to estimate fluoride intake from individual (e.g., water) or multiple (e.g., water and diet) exposure media)).<br>**Note:** The preferred analytical method to measure total fluoride levels in liquid samples is the ion selective electrode (ISE) method, with appropriate QA and calibration (e.g., standardized ionic strength buffer and control pH to ≤ 5). The ISE method is simple, sensitive, and rapid, and it is the most commonly used analytical method to measure fluoride in environmental and biological samples (ATSDR, 2003; WHO, 2004). The ISE method is reliable to about 0.019 mg/L, and it is the method recommended by NIOSH for measuring fluoride in urine (level of detection of 0.1 mg/L) (NRC, 2006; NIOSH, 1994).<br>**Note**: For fluoride levels in urine, a study needs to specify whether spot urine or 24 hour urine was used. For spot urine samples, a study should include an explanation for how urinary dilution was examined (e.g., specific gravity or creatinine). |

**Q8 Cross-sectional - Definitely Low Risk-of-bias (++)**

**Note:** May include other less commonly used methods, such as gas or liquid chromatography or colorimetric methods, as long as appropriate QA is employed and calibration is documented.

**Q8 Cross-sectional - Probably Low Risk-of-bias (+)**

Indirect evidence that the exposure or intake was consistently assessed using well-established methods that directly measure exposure or intake,

**OR** exposure was assessed using indirect measures (e.g., drinking water levels and residency, questionnaire or occupational exposure assessment by a certified industrial hygienist) that have been validated or empirically shown to be consistent with methods that directly measure exposure (i.e., inter-methods validation: one method vs. another),

**AND** there is sufficient range or variation in exposure measurements across groups to potentially identify associations with health outcomes (at a minimum from high exposure or ever exposed from low exposure or never exposed),

**AND** there is evidence that most of the exposure data measurements are above the limit of quantitation for the assay such that different exposure groups can be distinguished,

**AND** there is evidence (direct or indirect) that the study used appropriate calibration and QA procedures.

**Note:** Includes studies that measure fluoride in subjects' blood, serum, plasma, or fingernails because the relationship between levels of fluoride in drinking water and blood, plasma, or serum levels is less well-established (ATSDR, 2003).

**Note:** Includes studies that report intake based on some self-reported elements.

**Q8 Cross-sectional - Probably High Risk-of-bias (-) or (NR)**

Indirect evidence that the exposure or intake was assessed using poorly validated methods that directly measure exposure,

**OR** there is evidence that the exposure was assessed using indirect measures that have not been validated or empirically shown to be consistent with methods that directly measure exposure (e.g., a job-exposure matrix or self-report without validation) (record "NR" as basis for answer),

**OR** there is insufficient information provided about the exposure assessment, including validity and reliability, but no evidence for concern about the method used (record "NR" as basis for answer).

**Note:** Includes studies with ecological exposure metrics (e.g., few measurements from a large geographic area) for which there is evidence (indirect or direct) about migration between different geographic areas. If no information on migration is reported, then the geographic setting (rural vs. urban) should be considered. Insufficient information about migration may not be a large risk-of-bias concern for studies conducted in rural (low mobility/migration) areas, in contrast to studies conducted in urban (high migration/mobility) areas.

**Q8 Cross-sectional - Definitely High Risk-of-bias (--)**

Direct evidence that the exposure or intake was assessed using methods with poor validity,

**OR** evidence of exposure misclassification (e.g., differential recall of self-reported exposure, evidence for high population mobility that is not accounted for).

***9. Can we be confident in the outcome assessment?***

| **Q9 Cross-sectional - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that the neurobehavioral outcome was assessed using well-established, validated assessment methods (well-established test methods are listed in Table 5),<br>**AND** there is direct evidence that the outcome assessors (including study subjects, if outcomes were self-reported or reported by a parent or guardian) were adequately blinded to the exposure level, and it is unlikely that they could have broken the blinding prior to reporting outcomes,<br>**AND** there is direct evidence that the test methods are appropriate to the population being studied. Evidence can include: (1) the use of tests previously tested and validated in similar populations (e.g., the Raven's Test for Rural China applied in a study of Chinese schoolchildren) or (2) the authors provide direct evidence that the chosen methods had been specifically adapted for the study subjects and that results were valid and reproducible). |

| **Q9 Cross-sectional - Probably Low Risk-of-bias (+)** |
|---|
| Indirect evidence (see Note) that the outcome was assessed using instruments that were valid and reliable in the study population,<br>**OR** it is deemed that the outcome assessment methods used would not appreciably bias results,<br>**AND** there is indirect evidence that the outcome assessors were adequately blinded to the exposure level, and it is unlikely that they could have broken the blinding prior to reporting outcomes,<br>**OR** it is deemed that lack of adequate blinding of outcome assessors would not appreciably bias results (including that subjects self-reporting outcomes were likely not aware of reported links between the exposure and outcome lack of blinding is unlikely to bias a particular outcome).<br>**Note:** Indirect evidence includes: (1) the authors specify that they used methods listed in Table 5 or (2) the authors use instrument(s) not listed in Table 5 but indicate that they have been designed, tested, calibrated, or validated for measurement of relevant outcomes in the test subjects or a similar population. |

| **Q9 Cross-sectional - Probably High Risk-of-bias (-) or (NR)** |
|---|
| Indirect evidence (see Note) that the outcome assessment method is an insensitive or imprecise instrument,<br>**OR** there is indirect evidence that it was possible for outcome assessors to infer the exposure level prior to reporting outcomes (including that subjects self-reporting outcomes were likely aware of reported links between the exposure and outcome),<br>**OR** there is insufficient information provided about blinding of outcome assessors (record "NR" as basis for answer).<br>**Note:** Indirect evidence includes: (1) the authors specify that they used methods not listed in Table 5 and (2) the authors do not indicate (NR) that they have been designed, tested, calibrated, or validated for measurement of relevant outcomes in the test subjects or a similar population. |

| **Q9 Cross-sectional - Definitely High Risk-of-bias (--)** |
|---|
| Direct evidence (see Note) that the outcome assessment method is an insensitive or imprecise instrument,<br>**OR** there is direct evidence that the test method had not been previously calibrated or validated in similar populations, |

**Q9 Cross-sectional - Definitely High Risk-of-bias (--)**

**OR** there is direct evidence that outcome assessors were aware of the exposure level prior to reporting outcomes (including that subjects self-reporting outcomes were aware of reported links between the exposure and outcome).

**Note:** Direct evidence would include a previous demonstration that the instrument was not reliable in the study subjects or similar population or internal inconsistencies in the outcome assessment results or interpretation.

***10. Were all measured outcomes reported?***

**Q10 Cross-sectional - Definitely Low Risk-of-bias (++)**

Direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported. This would include outcomes reported with sufficient detail to be included in meta-analysis or fully tabulated during data extraction and analyses had been planned in advance.

**Q10 Cross-sectional - Probably Low Risk-of-bias (+)**

Indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported,

**OR** analyses that had not been planned in advance (i.e., retrospective unplanned subgroup analyses) are clearly indicated as such and deemed that unplanned analyses were appropriate and selective reporting would not appreciably bias results (e.g., appropriate analyses of an unexpected effect). This would include outcomes reported with insufficient detail such as only reporting that results were statistically significant (or not).

**Q10 Cross-sectional - Probably High Risk-of-bias (-) or (NR)**

Indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported,

**OR** and there is indirect evidence that unplanned analyses were included that may appreciably bias results,

**OR** there is insufficient information provided about selective outcome reporting ("NR" as basis for answer).

**Note:** Includes studies that report

**Q10 Cross-sectional - Definitely High Risk-of-bias (--)**

Direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported. In addition to not reporting outcomes, this would include reporting outcomes based on composite score without individual outcome components or outcomes reported using measurements, analysis methods or subsets of the data (e.g., subscales) that were not pre-specified or reporting outcomes not pre-specified, or that unplanned analyses were included that would appreciably bias results.

***11. Were there no other potential threats to internal validity?***

There are no fluoride-specific additions to the risk-of-bias questions for this evaluation. This question will be used to examine individual studies for appropriate statistical methods (e.g., confirmation of homogeneity of variance for ANOVA and other statistical tests that require normally distributed data). It will also be used for risk-of-bias considerations that do not fit under the other questions.

**Cohort studies**

***1. Was administered dose or exposure level adequately randomized? [NA]***

***2. Was allocation to study groups adequately concealed? [NA]***

***3. Did selection of study participants result in the appropriate comparison groups?***

| **Q3 Cohort - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that subjects (both exposed and non-exposed) were similar (e.g., recruited from the same eligible population, recruited using the same inclusion and exclusion criteria, and were of similar age, socioeconomic and health status), and recruited within the same time frame.<br>**Note:** A study will be generally be considered low risk-of-bias if baseline characteristics of groups differed but these differences were considered as potential confounding or stratification variables (see question #4). |

| **Q3 Cohort - Probably Low Risk-of-bias (+)** |
|---|
| Indirect evidence that subjects (both exposed and non-exposed) were similar (e.g., recruited from the same population, recruited with the same method of ascertainment using the same inclusion and exclusion criteria, and were of similar age and health status), recruited within the same time frame, and had similar participation/response rates,<br>**OR** differences between groups were not likely to substantively bias results.<br>**Note:** Includes studies where the authors state that characteristics of exposed and referent groups were similar (as above), but do not provide quantitative information on covariates. |

| **Q3 Cohort - Probably High Risk-of-bias (-) or (NR)** |
|---|
| Indirect evidence that subjects (both exposed and non-exposed) were not similar, were recruited within very different time frames, or had the very different participation/response rates,<br>**OR** there is insufficient information provided about the comparison group including a different rate of non-response without an explanation (record "NR" as basis for answer). |

| **Q3 Cohort - Definitely High Risk-of-bias (--)** |
|---|
| Direct evidence that subjects (both exposed and non-exposed) were not similar (e.g., recruited from the different eligible populations, recruited using different inclusion and exclusion criteria, or were significantly different in terms of age, socioeconomic, or health status), recruited within very different time frames, or had the very different participation/response rates. |

***4. Did study design or analysis account for important confounding and modifying variables?***

| **Q4 Cohort - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that appropriate adjustments or explicit considerations were made for the variables listed below as potential confounders and/or effect measure modifiers in the final analyses through the use of statistical models to reduce research-specific bias including standardization, matching, adjustment in multivariate models, stratification, or other methods that were appropriately justified. Acceptable consideration of appropriate adjustment factors includes |

**Q4 Cohort - Definitely Low Risk-of-bias (++)**

cases when the factor is not included in the final adjustment model because the author conducted analyses that indicated it did not need to be included,

**AND** there is direct evidence that primary covariates and confounders (including known neurodevelopmental toxicants lead and arsenic) were appropriately measured (using valid and reliable methods) and adjusted for,

**OR** there is direct evidence that certain covariates and cofounders that are anticipated to bias results were not present.

**Note:** The following variables should be considered as potential confounders and/or effect measure modifiers for the relationship between fluoride exposure and neurobehavioral outcomes**:** age, child's sex, race/ethnicity, maternal demographics (e.g., maternal age, mother's cohort, BMI parental behavioral and mental health disorders (e.g., ADHD, depression), socioeconomic status (e.g., maternal education, household income, marital status, crowding), smoking (e.g., maternal smoking status, secondhand tobacco smoke exposure), reproductive factors (e.g., parity), nutrition (e.g., BMI, growth, anemia), iodine deficiency/excess, mineral and other chemicals in water associated with neurotoxicity, maternal (and paternal) IQ, quantity and quality of caregiving environment (e.g., HOME score).

**Note:** Many studies report incidence of dental and/or skeletal fluorosis, and sometimes stratify results by fluorosis severity. Because fluorosis is highly correlated with fluoride exposure, one should consider how the fluorosis is handled in the study, especially if the study authors adjusted for fluorosis.

**Q4 Cohort - Probably Low Risk-of-bias (+)**

Indirect evidence that appropriate adjustments were made,

**AND** there is indirect evidence that potential covariates and confounders [age, child's sex, race/ethnicity, maternal demographics (e.g., maternal age, mother's cohort, BMI), parental behavioral and mental health disorders (e.g., ADHD, depression), socioeconomic status (e.g., maternal education, household income, marital status, crowding), smoking (e.g., maternal smoking status, secondhand tobacco smoke exposure), reproductive factors (e.g., parity), nutrition (e.g., BMI, growth, anemia), iodine deficiency/excess, mineral and other chemicals in water associated with neurotoxicity (e.g., arsenic and lead), maternal (and paternal) IQ, quantity and quality of caregiving environment (e.g., HOME score)] were appropriately measured and adjusted for,

**AND** there is evidence (direct or indirect) that covariates and confounders considered were assessed using valid and reliable measurements,

**OR** it is deemed that the covariate measures used would not appreciably bias results (i.e., the authors justify the validity of the measures from previously published research),

**AND** there is evidence (direct or indirect) that other covariates and confounders considered were not present or were appropriately adjusted for,

**OR** it is deemed that not considering or only considering a partial list of covariates or confounders in the final analyses would not appreciably bias results.

**Q4 Cohort - Probably High Risk-of-bias (-) or (NR)**

Indirect evidence that the distribution of important covariates and known confounders differed between the groups and was not appropriately adjusted for in the final analyses,

**OR** there is insufficient information provided about the distribution of known confounders (record "NR" as basis for answer),

| **Q4 Cohort - Probably High Risk-of-bias (-) or (NR)** |
|---|
| **OR** there is indirect evidence that there was an unbalanced distribution of co-exposures that could affect neurological development across the primary study groups, which was not appropriately adjusted for,<br>**OR** there is indirect evidence that covariates and confounders considered were assessed using measurements of unknown validity,<br>**OR** there is insufficient information provided about the measurement techniques used to assess covariates and confounders considered (record "NR" as basis for answer). |

| **Q4 Cohort - Definitely High Risk-of-bias (--)** |
|---|
| Direct evidence that the distribution of important covariates, known confounders, and co-exposures differed between the groups and was not accounted for,<br>**OR** confounding was demonstrated or likely to be present but not appropriately adjusted for in the final analyses,<br>**OR** there is direct evidence that covariates and confounders considered were assessed using non valid measurements,<br>**OR** there is indirect evidence of co-exposure to high levels of lead and arsenic (or other agents associated with negative effects on cognition) but these co-exposures were not appropriately measured and adjusted for.<br>**Note:** Includes studies that report high levels of skeletal fluorosis in the study population but do not adjust for it. |

***5. Were experimental conditions identical across study groups? [NA]***

***6. Were the research personnel blinded to the study group during the study? [NA]***

***7. Were outcome data complete without attrition or exclusion from analysis?***

| **Q7 Cohort - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that loss of subjects (i.e., incomplete outcome data) was adequately addressed and reasons were documented when human subjects were removed from a study.<br>**Note**: Acceptable handling of subject attrition includes: very little missing outcome data; reasons for missing subjects unlikely to be related to outcome (for survival data, censoring unlikely to be introducing bias); missing outcome data balanced in numbers across study groups, with similar reasons for missing data across groups,<br>**OR** missing data have been imputed using appropriate methods and characteristics of subjects lost to follow up or with unavailable records are described in identical way and are not significantly different from those of the study participants. |

| **Q7 Cohort - Probably Low Risk-of-bias (+)** |
|---|
| Indirect evidence that loss of subjects (i.e., incomplete outcome data) was adequately addressed and reasons were documented when human subjects were removed from a study,<br>**OR** it is deemed that the proportion lost to follow-up would not appreciably bias results. This would include reports of no statistical differences in characteristics of subjects lost to follow up or with unavailable records from those of the study participants. Generally, the higher the ratio of participants with missing data to participants with events, the greater potential there is for bias. For studies with a long duration of follow-up, some withdrawals for such reasons are inevitable. |

| **Q7 Cohort - Probably High Risk-of-bias (-) or (NR)** |
|---|
| Indirect evidence that loss of subjects (i.e., incomplete outcome data) was unacceptably large, or substantially different across groups, and not adequately addressed,<br>**OR** there is insufficient information provided about numbers of subjects lost to follow-up (record "NR" as basis for answer). |

| **Q7 Cohort - Definitely High Risk-of-bias (--)** |
|---|
| Direct evidence that loss of subjects (i.e., incomplete outcome data) was unacceptably large and not adequately addressed.<br>**Note**: Unacceptable handling of subject attrition includes: reason for missing outcome data likely to be related to true outcome, with either imbalance in numbers or reasons for missing data across study groups; or potentially inappropriate application of imputation. |

***8. Can we be confident in the exposure/intake characterization?***

| **Q8 Cohort - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that exposure or intake was consistently assessed (i.e., using the same method and during the same time-frame) using well-established methods that directly characterize exposure or intake,<br>**OR** fluoride intake is estimated from exposure sources, such as drinking water, that are well-characterized,<br>**OR** exposure was assessed using less-established methods that directly measure exposure and are validated against well-established methods,<br>**AND** there is sufficient range or variation in exposure measurements across groups to potentially identify associations with health outcomes,<br>**AND** there is evidence that most of the exposure data measurements are above the limit of quantitation for the assay such that different exposure groups can be distinguished,<br>**AND** there is evidence (direct or indirect) that the study used appropriate quality control (including blanks and spiked samples) for all analytical methods.<br>**Note:** Includes studies that measure fluoride in subjects' household drinking water or urine because the relationship between levels of fluoride in drinking water and urinary fluoride levels is generally well-characterized (ATSDR, 2003).<br>**Note:** Includes studies with direct intake estimates (e.g., studies that use high-quality measurements and validated estimation techniques to estimate fluoride intake from individual (e.g., water) or multiple (e.g., water and diet) exposure media)).<br>**Note:** The preferred analytical method to measure total fluoride levels in liquid samples is the ion selective electrode (ISE) method, with appropriate QA and calibration (e.g., standardized ionic strength buffer and control pH to ≤ 5). The ISE method is simple, sensitive, and rapid, and it is the most commonly used analytical method to measure fluoride in environmental and biological samples (ATSDR, 2003; WHO, 2004). The ISE method is reliable to about 0.019 mg/L, and it is the method recommended by NIOSH for measuring fluoride in urine (level of detection of 0.1 mg/L) (NRC, 2006; NIOSH, 1994).<br>**Note**: For fluoride levels in urine, a study needs to specify whether spot urine or 24 hour urine was used. For spot urine samples, a study should include an explanation for how urinary dilution was examined (e.g., specific gravity or creatinine).<br>**Note:** May include other less commonly used methods, such as gas or liquid chromatography or colorimetric methods, as long as appropriate QA is employed and calibration is documented. |

**Q8 Cohort - Probably Low Risk-of-bias (+)**

Indirect evidence that the exposure or intake was consistently assessed using well-established methods that directly measure exposure or intake,

**OR** exposure was assessed using indirect measures (e.g., drinking water levels and residency, questionnaire or occupational exposure assessment by a certified industrial hygienist) that have been validated or empirically shown to be consistent with methods that directly measure exposure (i.e., inter-methods validation: one method vs. another),

**AND** there is sufficient range or variation in exposure measurements across groups to potentially identify associations with health outcomes (at a minimum from high exposure or ever exposed from low exposure or never exposed),

**AND** there is evidence that most of the exposure data measurements are above the limit of quantitation for the assay such that different exposure groups can be distinguished,

**AND** there is evidence (direct or indirect) that the study used appropriate calibration and QA procedures.

**Note:** Includes studies that measure fluoride in subjects' blood, serum, plasma, or fingernails because the relationship between levels of fluoride in drinking water and blood, plasma, or serum levels is less well-established (ATSDR, 2003).

**Note:** Includes studies that report intake based on some self-reported elements

**Q8 Cohort - Probably High Risk-of-bias (-) or (NR)**

Indirect evidence that the exposure or intake was assessed using poorly validated methods that directly measure exposure,

**OR** there is evidence that the exposure was assessed using indirect measures that have not been validated or empirically shown to be consistent with methods that directly measure exposure (e.g., a job-exposure matrix or self-report without validation) (record "NR" as basis for answer),

**OR** there is insufficient information provided about the exposure assessment, including validity and reliability, but no evidence for concern about the method used (record "NR" as basis for answer).

**Note:** Includes studies with ecological exposure metrics (e.g., few measurements from a large geographic area) for which there is evidence (indirect or direct) about migration between different geographic areas. If no information on migration is reported, then the geographic setting (rural vs. urban) should be considered. Insufficient information about migration may not be a large risk-of-bias concern for studies conducted in rural (low mobility/migration) areas, in contrast to studies conducted in urban (high migration/mobility) areas.

**Q8 Cohort - Definitely High Risk-of-bias (--)**

Direct evidence that the exposure or intake was assessed using methods with poor validity,

**OR** evidence of exposure misclassification (e.g., differential recall of self-reported exposure, evidence for high population mobility that is not accounted for).

***9. Can we be confident in the outcome assessment?***

**Q9 Cohort - Definitely Low Risk-of-bias (++)**

Direct evidence that the neurobehavioral outcome was assessed using well-established, validated assessment methods (well-established test methods are listed in Table 5),

**AND** there is direct evidence that the outcome assessors (including study subjects, if outcomes were self-reported or reported by a parent or guardian) were adequately blinded to the exposure level, and it is unlikely that they could have broken the blinding prior to reporting outcomes,

**Q9 Cohort - Definitely Low Risk-of-bias (++)**

**AND** there is direct evidence that the test methods are appropriate to the population being studied. Evidence can include: (1) the use of tests previously tested and validated in similar populations (e.g., the Raven's Test for Rural China applied in a study of Chinese schoolchildren) or (2) the authors provide direct evidence that the chosen methods had been specifically adapted for the study subjects and that results were valid and reproducible).

**Q9 Cohort - Probably Low Risk-of-bias (+)**

Indirect evidence (see Note) that the outcome was assessed using instruments that were valid and reliable in the study population,

**OR** it is deemed that the outcome assessment methods used would not appreciably bias results,

**AND** there is indirect evidence that the outcome assessors were adequately blinded to the exposure level, and it is unlikely that they could have broken the blinding prior to reporting outcomes,

**OR** it is deemed that lack of adequate blinding of outcome assessors would not appreciably bias results (including that subjects self-reporting outcomes were likely not aware of reported links between the exposure and outcome lack of blinding is unlikely to bias a particular outcome).

**Note:** Indirect evidence includes: (1) the authors specify that they used methods listed in Table 5 or (2) the authors use instrument(s) not listed in Table 5 but indicate that they have been designed, tested, calibrated, or validated for measurement of relevant outcomes in the test subjects or a similar population.

**Q9 Cohort - Probably High Risk-of-bias (-) or (NR)**

Indirect evidence (see Note) that the outcome assessment method is an insensitive or imprecise instrument,

**OR** there is indirect evidence that it was possible for outcome assessors to infer the exposure level prior to reporting outcomes (including that subjects self-reporting outcomes were likely aware of reported links between the exposure and outcome),

**OR** there is insufficient information provided about blinding of outcome assessors (record "NR" as basis for answer).

**Note:** Indirect evidence includes: (1) the authors specify that they used methods not listed in Table 5 and (2) the authors do not indicate (NR) that they have been designed, tested, calibrated, or validated for measurement of relevant outcomes in the test subjects or a similar population.

**Q9 - Cohort Definitely High Risk-of-bias (--)**

Direct evidence (see Note) that the outcome assessment method is an insensitive or imprecise instrument, **OR** there is direct evidence that the test method had not been previously calibrated or validated in similar populations,

**OR** there is direct evidence that outcome assessors were aware of the exposure level prior to reporting outcomes (including that subjects self-reporting outcomes were aware of reported links between the exposure and outcome).

**Note:** Direct evidence would include a previous demonstration that the instrument was not reliable in the study subjects or similar population or internal inconsistencies in the outcome assessment results or interpretation.

***10. Were all measured outcomes reported?***

| **Q10 Cohort - Definitely Low Risk-of-bias (++)** |
|---|
| Direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported. This would include outcomes reported with sufficient detail to be included in meta-analysis or fully tabulated during data extraction and analyses had been planned in advance. |

| **Q10 Cohort - Probably Low Risk-of-bias (+)** |
|---|
| Indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported,<br>**OR** analyses that had not been planned in advance (i.e., retrospective unplanned subgroup analyses) are clearly indicated as such and deemed that unplanned analyses were appropriate and selective reporting would not appreciably bias results (e.g., appropriate analyses of an unexpected effect). This would include outcomes reported with insufficient detail such as only reporting that results were statistically significant (or not). |

| **Q10 Cohort - Probably High Risk-of-bias (-) or (NR)** |
|---|
| Indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported,<br>**OR** and there is indirect evidence that unplanned analyses were included that may appreciably bias results,<br>**OR** there is insufficient information provided about selective outcome reporting (record "NR" as basis for answer). |

| **Q10 Cohort - Definitely High Risk-of-bias (--)** |
|---|
| Direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported. In addition to not reporting outcomes, this would include reporting outcomes based on composite score without individual outcome components or outcomes reported using measurements, analysis methods or subsets of the data (e.g., subscales) that were not pre-specified or reporting outcomes not pre-specified, or that unplanned analyses were included that would appreciably bias results. |

***11. Were there no other potential threats to internal validity?***

There are no fluoride-specific additions to the risk-of-bias questions for this evaluation. This question will be used to examine individual studies for appropriate statistical methods (e.g., confirmation of homogeneity of variance for ANOVA and other statistical tests that require normally distributed data). It will also be used for risk-of-bias considerations that do not fit under the other questions.

### *Experimental Animal Studies*

***1. Was administered dose or exposure level adequately randomized?***

| **Q1 Experimental Animal - Definitely Low Risk-of-bias (++)** |
|---|
| **Study provides:** Direct evidence that animals were allocated to all study groups, including concurrent controls, using a method with a random component,<br>**AND** there is direct evidence that the study used a concurrent control group as an indication that randomization covered all study groups,<br>**Note:** Acceptable methods of randomization include: referring to a random number table, using a computer random number generator, coin tossing, or shuffling cards (JPT Higgins and S Green 2011).<br>**Note:** Restricted randomization (e.g., blocked randomization) to ensure particular allocation ratios will be considered low bias. Similarly, stratified randomization approaches that attempt to minimize imbalance between groups on important prognostic factors (e.g., body weight) will be considered acceptable. |

| **Q1 Experimental Animal - Probably Low Risk-of-bias (+)** |
|---|
| **Study provides:** Indirect evidence that animals were allocated to all study groups including concurrent controls using a method with a random component (i.e., authors state random allocation, without description of method),<br>**AND either**:<br>• Evidence that the study used a concurrent control group as an indication that randomization covered all study groups,<br>**OR**<br>• It is deemed that allocation without a clearly random component would not appreciably bias results. |

| **Q1 Experimental Animal - Probably High Risk-of-bias (-) or (NR)** |
|---|
| **Study provides either**:<br>• Indirect evidence that animals were allocated to all study groups using a method with a non-random component,<br>**OR**<br>• Indirect evidence that there was a lack of a concurrent control group,<br>**OR**<br>• There is insufficient information provided about how animals were allocated to study groups (record "NR" as basis for answer). |

| **Q1 Experimental Animal - Definitely High Risk-of-bias (--)** |
|---|
| **Study provides either**:<br>• Direct evidence that animals were allocated to all study groups using a non-random method including judgment of the investigator, the results of a laboratory test, or a series of tests,<br>**OR**<br>• Direct evidence that there was a lack of a concurrent control group. |

***2. Was allocation to study groups adequately concealed?***

| Q2 Experimental Animal - Definitely Low Risk-of-bias (++) |
|---|
| **Study provides:** Direct evidence that at the time of assigning study groups the research personnel did not know what group animals were allocated to, and it is unlikely that they could have broken the blinding of allocation until after assignment was complete and irrevocable.<br>**Note:** Acceptable methods used to ensure allocation concealment include sequentially numbered treatment containers of identical appearance or equivalent methods. |

| Q2 Experimental Animal - Probably Low Risk-of-bias (+) |
|---|
| **Study provides either**:<br>• Indirect evidence that at the time of assigning study groups the research personnel did not know what group animals were allocated to and it is unlikely that they could have broken the blinding of allocation until after assignment was complete and irrevocable,<br>**OR**<br>• It is deemed that lack of adequate allocation concealment would not appreciably bias results. |

| Q2 Experimental Animal - Probably High Risk-of-bias (-) or (NR) |
|---|
| **Study provides either**:<br>• Indirect evidence that at the time of assigning study groups it was possible for the research personnel to know what group animals were allocated to, or it is likely that they could have broken the blinding of allocation before assignment was complete and irrevocable,<br>**OR**<br>• There is insufficient information provided about allocation to study groups (record "NR" as basis for answer). |

| Q2 Experimental Animal - Definitely High Risk-of-bias (--) |
|---|
| **Study provides:** Direct evidence that at the time of assigning study groups it was possible for the research personnel to know what group animals were allocated to, or it is likely that they could have broken the blinding of allocation before assignment was complete and irrevocable. |

***3. Did selection of study participants result in the appropriate comparison groups? [NA]***

***4. Did study design or analysis account for important confounding and modifying variables? [NA]***

***5. Were experimental conditions identical across study groups?***

| Q5 Experimental Animal - Definitely Low Risk-of-bias (++) |
|---|
| **Study provides:** Direct evidence that same vehicle was used in control and experimental animals,<br>**AND** direct evidence that non-treatment-related experimental conditions were identical across study groups (i.e., the study explicitly states that animals were all in the same room or provides other details to indicate that the conditions were identical).<br>**Note:** In many cases the vehicle may just be drinking water |

| Q5 Experimental Animal - Probably Low Risk-of-bias (+) |
|---|
| **Study provides either**:<br>• Indirect evidence that the same vehicle was used in control and experimental animals,<br>**OR**<br>• It is deemed that the vehicle used would not appreciably bias results, |

**Q5 Experimental Animal - Probably Low Risk-of-bias (+)**

**AND** the authors do not explicitly state that non-treatment-related experimental conditions were identical (e.g., experimental conditions were provided, but there is no statement or demonstration that conditions were the same across study groups)

**Q5 Experimental Animal - Probably High Risk-of-bias (-) or (NR)**

**Study provides either**: Indirect evidence that the vehicle differed between control and experimental animals,

**OR**

- Authors did not report the vehicle used (record "NR" as basis for answer),

**OR**

- Indirect evidence that non-treatment-related experimental conditions were not comparable between study groups.

**Q5 Experimental Animal - Definitely High Risk-of-bias (--)**

**Study provides either**:

- Direct evidence from the study report that control animals were untreated, or treated with a different vehicle than experimental animals,

**OR**

- Direct evidence that non-treatment-related experimental conditions were not comparable between study groups.

***6. Were the research personnel blinded to the study group during the study?***

**Q6 Experimental Animal - Definitely Low Risk-of-bias (++)**

**Study provides:** Direct evidence that the research personnel were adequately blinded to study group, and it is unlikely that they could have broken the blinding during the study.

**Note:** Methods used to ensure blinding include: central allocation, sequentially numbered treatment containers of identical appearance, sequentially numbered animal cages; or equivalent methods.

**Q6 Experimental Animal - Probably Low Risk-of-bias (+)**

**Study provides either**:

- Indirect evidence that the research personnel were adequately blinded to study group, and it is unlikely that they could have broken the blinding during the study,

**OR**

- It is deemed that lack of adequate blinding during the study would not appreciably bias results. This would include cases where blinding was not possible but research personnel took steps to minimize potential bias, such as restricting the knowledge of study group to veterinary or supervisory personnel monitoring for overt toxicity, or randomized husbandry or handling practices (e.g., placement in the animal room, necropsy order, etc.).

**Q6 Experimental Animal - Probably High Risk-of-bias (-) or (NR)**

**Study provides either**:

- Indirect evidence that the research personnel were not adequately blinded to study group,

**OR**

- There is insufficient information provided about blinding to study group during the study (record "NR" as basis for answer).

**Q6 Experimental Animal - Definitely High Risk-of-bias (--)**

Direct evidence that the research personnel were not adequately blinded to study group.

***7. Were outcome data complete without attrition or exclusion from analysis?***

**Q7 Experimental Animal - Definitely Low Risk-of-bias (++)**

**Study provides either**:

- Direct evidence that loss of animals was adequately addressed and reasons were documented when animals were removed from a study.

**OR**

- Direct evidence that missing data have been imputed using appropriate methods (insuring that characteristics of animals are not significantly different from animals retained in the analysis).

**Note**: Acceptable handling of attrition includes: very little missing outcome data; reasons for missing animals unlikely to be related to outcome (or for survival data, censoring unlikely to be introducing bias); missing outcome data balanced in numbers across study groups, with similar reasons for missing data across groups; missing outcomes is not enough to impact the effect estimate.

**Q7 Experimental Animal - Probably Low Risk-of-bias (+)**

**Study provides either**:

- Indirect evidence that loss of animals was adequately addressed and reasons were documented when animals were removed from a study,

**OR**

- It is deemed that the proportion lost would not appreciably bias results. This would include reports of no statistical differences in characteristics of animals removed from the study from those remaining in the study.

**Q7 Experimental Animal - Probably High Risk-of-bias (-) or (NR)**

**Study provides either**:

- Indirect evidence that loss of animals was unacceptably large and not adequately addressed,

**OR**

- There is insufficient information provided about loss of animals (record "NR" as basis for answer).

**Q7 Experimental Animal - Definitely High Risk-of-bias (--)**

**Study provides:** Direct evidence that loss of animals was unacceptably large and not adequately addressed.

**Note**: Unacceptable handling of attrition or exclusion includes: reason for loss is likely to be related to true outcome, with either imbalance in numbers or reasons for loss across study groups.

***8. Can we be confident in the exposure characterization?***

**Q8 Experimental Animal - Definitely Low Risk-of-bias (++)**

**Study provides:** Direct evidence that the exposure to fluoride was independently characterized and purity confirmed generally as ≥98%, (and compliance with the treatment, if applicable)

**AND** exposure was consistently administered (i.e., with the same method and time-frame) across treatment groups,

**Q8 Experimental Animal - Definitely Low Risk-of-bias (++)**

**AND** for dietary or drinking water studies information is provided on consumption or internal dose metrics to confirm expected exposure levels sufficiently to allow discrimination between exposure groups,

**AND** if internal dose metrics are available, there is direct evidence that most of the exposure data measurements are above the limit of quantitation for the assay such that different exposure groups can be distinguished,

**AND** if internal dose metrics are available, the study used spiked samples or a dilution curve to confirm assay performance,

**AND** the analytical methods used to independently characterize fluoride are described or referenced.

**Note:** If controls are administered tap water, the level of fluoride in the drinking water should be provided.

**Note:** The preferred analytical method to measure total fluoride levels is the ion selective electrode (ISE) method, with appropriate QA and calibration (e.g., standardized ionic strength buffer and control pH to ≤ 5). The ISE method is simple, sensitive, and rapid, and it is the most commonly used analytical method to measure fluoride in environmental and biological samples (ATSDR 2003, WHO 2004). The ISE method is reliable to about 0.019 mg/L, and it is the method recommended by NIOSH for measuring fluoride in urine (level of detection of 0.1 mg/L) (NIOSH 1994, NRC 2006).

**Note:** Includes other less-established (or not commonly used) methods, such as gas or liquid chromatography, colorimetric methods, or the acid-hexamethyldisiloxane diffusion method, as long as appropriate QA and calibration were well-documented.

**Note:** If internal dose measurements are made, measurement of fluoride in blood, serum, plasma, bone, or in urine are the standard accepted biomarkers of exposure.

**Note**: For internal dose metrics, the timing of the fluoride exposure assessment (sample collection) in relation to treatment (e.g., at end of period, mid-way, at outcome assessment) should be provided. The internal dose should be measured at a time to represent the exposure, therefore, measuring internal dose in close temporal proximity to the outcome assessment may not be appropriate if the outcome is measured months after the exposure.

**Q8 Experimental Animal - Probably Low Risk-of-bias (+)**

**Study provides**: Indirect evidence that the exposure to fluoride was appropriately characterized and purity confirmed generally as ≥98% (i.e., the supplier of the chemical provides documentation of the purity of the chemical) **OR** direct evidence that purity was independently confirmed as ≥95% and it is deemed that impurities of up to 5% would not appreciably bias results,

**AND** exposure was consistently administered (i.e., with the same method and time-frame) across treatment groups,

**AND** for dietary or drinking water studies, no information is provided on consumption or internal dose metrics,

**AND** if internal dose metrics are available, there is indirect evidence that most of the exposure data measurements are above the limit of quantitation for the assay such that different exposure groups can be distinguished.

**Note**: Studies without purity, stability, or consumption information can still be considered to be probably low risk-of-bias if there are internal measurements that indicate there is low concern for bias.

**Q8 Experimental Animal - Probably Low Risk-of-bias (+)**

**Note**: Fluorosilicic acid is generally provided as a 20% weight volume solution in water. This is acceptable because it is assumed that they used a compound of appropriate purity to create the solution and the dissociation of the fluoride ion is complete.

**Q8 Experimental Animal - Probably High Risk-of-bias (-) or (NR)**

**Study provides either**:

- Indirect evidence that the exposure (including purity of the test substance and compliance with the treatment, if applicable) was assessed using poorly validated methods,

**OR**

- There is insufficient information provided about the validity of the exposure assessment method, but no evidence for concern (record "NR" as basis for answer),

**AND** if internal dose metrics are available, there is indirect evidence that most of the exposure data measurements are below the limit of quantitation for the assay such that different exposure groups cannot be distinguished.

**Q8 Experimental Animal - Definitely High Risk-of-bias (--)**

**Study provides**: Direct evidence that the exposure (including purity of the test substance and compliance with the treatment, if applicable) was assessed using poorly validated methods.

***9. Can we be confident in the outcome assessment?***

**Q9 Experimental Animal - Definitely Low Risk-of-bias (++)**

**Study provides:** Direct evidence that the outcome was assessed using well-established methods and assessed at the same length of time after initial exposure in all study groups,

**AND either**:

- Direct evidence that the outcome assessors were adequately blinded to the study group, and it is unlikely that they could have broken the blinding prior to reporting outcomes,

**OR**

- Outcomes were assessed with a fully automated method (e.g., automatic video recording and scoring of behavioral performance), which removes the potential bias of outcome assessors (note: a semi-automated method such as video recording without the automated scoring is not considered fully automated)

**Note:** Well-established methods will depend on the outcome, but examples of such methods may include: Morris water maze, T-maze, Y-maze, novel object recognition, mini-holeboard activity, activity cage, step-down test, shuttle box, operant behavior tests, open field, plank walking, rotarod, slanted surface, auditory startle, negative geotaxis, tail immersion, Von Frey hair test, cliff avoidance, surface righting, pivoting/orienting reflex, forced swim test, tail suspension test, and the elevated plus maze.

**Note:** There are standard protocols for each of these well-established methods. For example, the general protocol for the Morris water maze includes a task acquisition phase, during which the animal learns the location of a hidden platform over successive training sessions with multiple trials per day, followed by a probe test to measure spatial memory for the hidden platform location. Studies that use the Morris water maze should report performance in both the task acquisition phase and the probe test, and large deviations from this general protocol should be documented and supported by previously published studies.

**Q9 Experimental Animal - Probably Low Risk-of-bias (+)**

**Study provides either**:

- Indirect evidence that the outcome was assessed using acceptable methods (i.e., deemed valid and reliable but not the gold standard) **AND** indirect evidence that the outcome was assessed at the same length of time after initial exposure in all study groups,

**OR**

- It is deemed that the outcome assessment methods used would not appreciably bias results,

**AND either**:

- Indirect evidence that the outcome assessors were adequately blinded to the study group, and it is unlikely that they could have broken the blinding prior to reporting outcomes,

**OR**

- It is deemed that lack of adequate blinding of outcome assessors would not appreciably bias results, which is more likely to apply to objective outcome measures.

**Note:** For some outcomes, particularly histopathology assessment, outcome assessors are not blind to study group as they require comparison to the control to appropriately judge the outcome, but additional measures such as multiple levels of independent review by trained pathologists can minimize potential bias.

**Q9 Experimental Animal - Probably High Risk-of-bias (-) or (NR)**

**Study provides either**:

- Indirect evidence that the outcome assessment method is an insensitive instrument,

**OR**

- Indirect evidence that the length of time after initial exposure differed by study group,

**OR**

- Indirect evidence that it was possible for outcome assessors to infer the study group prior to reporting outcomes without sufficient quality control measures,

**OR**

- There is insufficient information provided about blinding of outcome assessors (record "NR" as basis for answer).

**Q9 Experimental Animal - Definitely High Risk-of-bias (--)**

**Study provides either**:

- Direct evidence that the outcome assessment method is an insensitive, or internally or externally invalid instrument,

**OR**

- Direct evidence that the length of time after initial exposure differed by study group,

**OR**

- Direct evidence for lack of adequate blinding of outcome assessors, including no blinding or incomplete blinding without quality control measures.

***10. Were all measured outcomes reported?***

**Q10 Experimental Animal - Definitely Low Risk-of-bias (++)**

**Study provides**: Direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported. This would include outcomes reported with sufficient detail to

| Q10 Experimental Animal - Definitely Low Risk-of-bias (++) |
|---|
| be included in meta-analysis or fully tabulated during data extraction and analyses had been planned in advance. |

| Q10 Experimental Animal - Probably Low Risk-of-bias (+) |
|---|
| **Study provides either**:<br>• Indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have been reported,<br>**OR**<br>• Indirect evidence that analyses that had not been planned in advance (i.e., retrospective unplanned subgroup analyses) are clearly indicated as such and deemed that unplanned analyses were appropriate and selective reporting would not appreciably bias results (e.g., appropriate analyses of an unexpected effect). This would include outcomes reported with insufficient detail such as only reporting that results were statistically significant (or not). |

| Q10 Experimental Animal - Probably High Risk-of-bias (-) or (NR) |
|---|
| **Study provides either**:<br>• Indirect evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported,<br>**OR**<br>• Indirect evidence that unplanned analyses were included that may appreciably bias results,<br>**OR**<br>• There is insufficient information provided about selective outcome reporting (record "NR" as answer basis). |

| Q10 Experimental Animal - Definitely High Risk-of-bias (--) |
|---|
| **Study provides:** Direct evidence that all of the study's measured outcomes (primary and secondary) outlined in the protocol, methods, abstract, and/or introduction (that are relevant for the evaluation) have not been reported. In addition to not reporting outcomes, this would include reporting outcomes based on composite score without individual outcome components or outcomes reported using measurements, analysis methods or subsets of the data (e.g., subscales) that were not pre-specified or reporting outcomes not pre-specified, or that unplanned analyses were included that would appreciably bias results. |

***11. Were there no other potential threats to internal validity?***

Internal validity refers to whether the methods and modes of analysis used in a study can be interpreted to reflect a potential causal relationship between specific factor(s) and observed outcomes. It can be interpreted to mean, generally, whether a study has been done "right" so that the results are "valid" in the specific study setting and is free of bias and confounding. This question will be used to examine individual studies for appropriate statistical methods (e.g., confirmation of homogeneity of variance for ANOVA and other statistical tests that require normally distributed data). It will also be used for risk-of-bias considerations that do not fit under the other questions.

**Q11 Experimental Animal - Definitely Low Risk-of-bias (++)**

**Study provides:** Direct evidence that homogeneity of variance was tested for any statistical test that requires normally distributed data (e.g., t-test, ANOVA).

**AND** Direct evidence that repeated measures statistical analyses were used for any experiments that repeatedly measured outcomes in the same animals,

**AND** Direct evidence that the litter was considered the basic unit of analysis for any study that used littermates in an experiment.

**Note:** Due to differences between males and females, it is preferable that results for males and females be reported separately. Reporting the results together can bias the results towards the null if an effect was observed in only one sex.

**Q11 Experimental Animal - Probably Low Risk-of-bias (+)**

**Study provides:** Indirect evidence that the litter was considered the basic unit of analysis for any study that used littermates in an experiment.

**AND** indirect evidence that repeated measures statistical analyses were used for any experiments that repeatedly measured outcomes in the same animals.

**Q11 Experimental Animal - Probably High Risk-of-bias (-) or (NR)**

**Study provides either:**

- Indirect evidence that homogeneity of variance was not tested for any statistical test that requires normally distributed data (e.g., t-test, ANOVA),

**OR**

- Indirect evidence that repeated measures statistical analyses were not used for any experiments that repeatedly measured outcomes in the same animals,

**OR**

- Indirect evidence that the litter was not considered the basic unit of analysis for any study that used littermates in an experiment.

**OR**

- There is insufficient information provided about statistical methods including if litter was used as the basic unit (record “NR” as basis for answer).

**Q11 Experimental Animal - Definitely High Risk-of-bias (--)**

**Study provides either:**

- Direct evidence that homogeneity of variance was not tested for any statistical test that requires normally distributed data (e.g., t-test, ANOVA),

**OR**

- Direct evidence that repeated measures statistical analyses were not used for any experiments that repeatedly measured outcomes in the same animals,

**OR**

- Direct evidence that the litter was not considered the basic unit of analysis.

**Note**: Includes studies that considered each littermate an independent observation and the individual pup as the experimental unit.