Review Article

Mixture Toxicity: Evidence from Experimental Studies on Concurrent Chemical Exposures

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This review synthesizes evidence from studies investigating the toxicological consequences of concurrent chemical exposures, emphasizing the inadequacy of traditional single-chemical risk assessment models. Organisms inhabiting natural environments are frequently exposed to complex mixtures of chemicals, leading to interactions that often produce non-additive effects such as synergism or antagonism, rather than simple additive responses predicted by conventional toxicological models. Experimental studies in animals and aquatic models have demonstrated that such exposures can be influenced by multiple variables including chemical identity, dose ratios, exposure duration, biological endpoints, and mechanistic pathways. The present review highlights key methodological approaches, such as concentration addition (CA) and independent action (IA) models, which attempt to predict mixture toxicity, though their reliability varies considerably. Critical factors like exposure timing and the biological characteristics of test organisms further complicate predictions. The translational challenges of extrapolating findings from animal models to humans, given species-specific toxicokinetic and genetic differences, are also explored. To address these complexities, this paper advocates for mechanistically-informed frameworks that incorporate high-throughput omics technologies, computational modeling, and standardized protocols for assessing environmentally relevant mixtures. It calls for a shift toward tiered, cumulative risk-assessment strategies that reflect real-world exposure scenarios and prioritize vulnerable populations. Such a transition is essential for advancing predictive toxicology and improving public and environmental health protection. The review ultimately calls for abandoning the outdated single-agent paradigm in favor of holistic, evidence-based approaches capable of managing the complexity of chemical exposures.

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Abbreviations

ADME: Absorption, Distribution, Metabolism, and Excretion AOP: Adverse Outcome Pathway CA: Concentration Addition CRA: Cumulative Risk Assessment IA: Independent Action MoA: Mode of Action NOAEL: No Observed Adverse Effect Level PFAS: Per- and Polyfluoroalkyl Substances PFOS: Perfluorooctane Sulfonate PFOA: Perfluorooctanoic Acid PBTK: Physiologically-Based Toxicokinetic TSCA: Toxic Substances Control Act US EPA: United States Environmental Protection Agency

Introduction

The biosphere is increasingly saturated with a vast and heterogeneous array of anthropogenic chemicals originating from industrial activities, agriculture, pharmaceuticals, consumer products, and natural processes. This omnipresent chemical landscape has created a scenario in which virtually all living organisms are continuously and concurrently exposed to complex mixtures of toxicants rather than isolated substances[1][2]. Environmental and occupational exposures rarely involve single chemicals but rather complex mixtures that can interact through various toxicological mechanisms. Understanding these interactions is basic for accurate risk assessment and regulatory decision-making.

The traditional approach of assessing individual chemicals, which may underestimate or overestimate the risks posed by concurrent exposures, requires the development of mixture-specific assessment methodologies[3][4]. Single-chemical approaches often mischaracterize health risks, as chemicals within mixtures can interact in additive, synergistic, or antagonistic ways[5][6][7]. These interactive effects are not arbitrary but are shaped by multiple interrelated factors, including the physicochemical properties of the compounds, the relative concentrations, timing, sequence, and duration of exposure, as well as the biological characteristics of the exposed organism[8][9][10].

Experimental animal studies offer a controlled, ethically managed, and mechanistically informative platform to elucidate the nature and impact of such interactions. These models enable dissection of joint toxic actions, identification of vulnerable developmental windows, such as gestation, lactation, and early postnatal life, as well as the exploration of mechanisms ranging from molecular disruptions to physiological alterations[11][12][13][14][15][16][17]. Moreover, animal studies facilitate the generation of crucial dose-response data for mixtures, support physiologically based toxicokinetic (PBTK)

modeling[18] and contribute directly to the refinement of cumulative risk assessment methodologies[19][20].

The translational relevance of such findings is underscored by growing evidence linking mixture exposures in animal models to pathologies analogous to those observed in humans, such as liver damage from metal mixtures[21][22], reinforcing their role in shaping more ecologically and clinically relevant regulatory policies[23][4].

Considering the above, the purpose of this review was to synthesize and critically evaluate the current body of experimental studies on the toxicological effects of chemical mixtures. The specific objectives are: (1) to systematically summarize key findings from a wide range of studies, focusing on the types and mechanisms of observed interactions (additivity, synergy, antagonism); (2) to analyze the influence of critical variables such as dose, timing, mixture composition, and biological susceptibility; (3) to discuss methodological strengths and limitations in current experimental and modeling approaches; and (4) to identify knowledge gaps and propose future research directions aimed at enhancing the scientific basis for assessing and managing risks associated with real-world chemical co-exposures.

Methods (Search Strategy)

This review manuscript was meticulously compiled through an iterative and expansive literature search strategy designed to identify and incorporate a broad spectrum of experimental studies investigating the cumulative, interactive, or combined effects resulting from concurrent or closely sequential exposure to two or more distinct chemical toxicants. A systematic search was conducted across primary scientific databases, including PubMed, Scopus, Web of Science, and Google Scholar, utilizing a set of search terms and Boolean operators, including but not limited to "chemical mixtures," "combined exposure," "concurrent exposure," "cumulative toxicity," "toxicant interactions," "joint action," "synergism," "antagonism," and "additivity," paired with "animal models" and "experimental studies," frequently combined with specific model organisms (e.g., "mice," "rats," "Daphnia magna," "zebrafish," "rabbits," "primates") and pertinent chemical classes or individual toxicants of interest (e.g., "metals," "pesticides," "organophosphates," "pyrethroids," "endocrine disruptors," "solvents," "nanomaterials," "air pollutants," "lead," "mercury," "arsenic," "cadmium," "bisphenol A," "phthalates," "PCBs").

Stringent inclusion criteria were applied for primary studies emphasizing original in vivo research conducted using established animal models (both vertebrate and invertebrate) that reported quantifiable

toxicological outcomes (e.g., mortality, morbidity, developmental effects, neurobehavioral changes, organ-specific pathology, biochemical alterations, genotoxicity, carcinogenicity), resulting from well-defined co-exposures.

Review articles, methodological papers, perspective pieces, and risk assessment framework documents, were strategically employed to frame the introductory and discussion sections, provide essential theoretical and mechanistic background, and to understand the historical evolution and current challenges in assessing risks from chemical mixtures.

Methodological Approaches and Theoretical Frameworks

Dawson et al.[24] established fundamental principles for evaluating mixture toxicity through their examination of 30 binary combinations of soft electrophiles using dose-response curve analysis with the *Vibrio fischeri* bioluminescence inhibition assay (Microtox test). It was demonstrated that time-dependent toxicity tests were essential for understanding mixture interactions, as chemicals with fully irreversible effects behaved differently from those with partially or fully reversible toxic effects. The study revealed that while most combined effects were close to dose-additive for hazard assessment purposes, particularly for congeneric chemicals sharing similar reactive mechanisms, the underlying mechanisms were considerably more complex than simple additivity models would suggest. Extending that initial work, Rice et al.[20] proposed an iterative, practical approach for assessing human exposures to chemical mixtures present in the environment, encompassing both Problem Formulation and Exposure Assessment elements. This methodology emphasized the importance of understanding changes in mixture composition due to differential transport, degradation, and partitioning processes in the environment, focusing on methods for identifying co-occurring chemicals, characterizing exposure levels and patterns, and grouping chemicals for cumulative assessment. While primarily an exposure assessment framework, it directly informs the design of relevant animal mixture studies by helping to prioritize mixtures of concern and define realistic exposure scenarios.

Sasso et al.[18] developed the Generalized Toxicokinetic Modeling system for Mixtures (GTMM), a physiologically-based framework that maintains consistency across different chemicals while incorporating interaction effects of complex mixtures, specifically designed for chemical mixtures containing metals. This sophisticated computational framework allows for the simulation of complex absorption, distribution, metabolism, and excretion (ADME) processes for multiple metals concurrently, accounting for potential interactions at the kinetic level, such as competition for transporters or binding

sites. This system demonstrated the feasibility of modeling toxicokinetics of complex, interacting mixtures based on available literature information, providing a more mechanistic basis for assessing mixture toxicity. The methodological landscape was further advanced by Hertzberg et al.[25], who presented a four-step approach for evaluating chemical mixture data consistency with dose addition. Their framework evaluated toxic proportionality, mixture model fit, agreement between mixture data and combined prediction models, and consistency between theoretical and observed mixture effects. This approach provides researchers and regulators with a systematic methodology for evaluating whether empirically observed mixture toxicity data conform to the dose addition model, the prevailing default assumption in mixture risk assessment, or exhibit significant deviations indicative of synergistic or antagonistic interactions. The framework employs rigorous statistical comparisons between experimentally derived mixture dose-response relationships and theoretical dose-response curves predicted under the dose addition assumption, thereby enabling quantitative characterization of interaction types and precise determination of their magnitude relative to additivity baselines. That analysis demonstrated that statistical adjustment for multiple comparisons is necessary due to the increased opportunities to reject dose addition hypotheses. Similarly, Leeman et al.[26] addressed the safety assessment framework based on the Threshold of Toxicological Concern, reviewing evidence suggesting that even at low doses (often below NOAELs), mixtures of chemicals, particularly those sharing common modes of action or affecting common biological pathways, could elicit significant cumulative effects. It was concluded that cumulative effects at exposure levels below established thresholds might occur in complex mixtures, emphasizing the "something from nothing" phenomenon. In turn, Gallagher et al.[27] reviewed ten US EPA cumulative risk assessments (CRAs), identifying key lessons for implementation. CRAs, which assess risks from multiple chemical and non-chemical stressors, require iterative approaches, stakeholder engagement, and tiered strategies to address multiroute exposures and geographical scales. These assessments enhance the real-world relevance of animal studies by prioritizing realistic exposure scenarios and vulnerable populations, thereby informing mixture toxicity research design.

On the other hand, the role of mode of action (MoA) in predicting mixture toxicity has been extensively studied. Verhaar et al.[28] proposed a classification system for environmental pollutants based on their MoA, which has been widely used in ecological risk assessment. Russom et al.[29] developed a method for predicting modes of toxic action from chemical structure, which has been applied to various aquatic species. More recently, Bauer et al.[30] and Sapounidou[31] proposed new classification algorithms based

on molecular initiating events, providing a more mechanistic basis for predicting mixture toxicity. In turn, the European Food Safety Authority (EFSA) has also contributed to the development of component-based approaches for human risk assessment of multiple chemicals[32]. These approaches consider the toxicological properties of individual components and their potential interactions, providing a more comprehensive assessment of mixture toxicity.

Influence of Environmental Stressors

The role of concurrent stressors in modulating mixture toxicity represents an important but understudied area[33]. Maternal restraint stress in mammals has been linked to negative outcomes such as impaired implantation, reduced embryo and fetal survival, lower birth weights, skeletal abnormalities, and alterations in neurobehavioral development. Given that pregnant women may simultaneously be exposed to both environmental metals and various forms of stress, Domingo et al.[34] reviewed the influence of maternal stress on metal-induced pre- and postnatal effects. Experimental studies were conducted in our laboratory. Thus, Colomina et al.[13] demonstrated that maternal restraint stress could substantially worsen the harmful impacts of toxic elements, though the magnitude of these interactions varied between mercury and arsenic exposures. Developmental landmarks showed significant delays when arsenite exposure was combined with restraint stress, while mercury-induced effects were less influenced by concurrent stress. Additional research by Colomina et al.[14], examining caffeine and aspirin combinations with maternal stress, revealed that prenatal stress could slightly exacerbate maternal and developmental toxicity of these drug combinations. These studies highlighted that environmental and physiological stressors could act as modulating factors in mixture toxicity, thereby complicating risk assessment predictions based solely on chemical interactions.

Further evidence of metal-stress interactions was shown by Torrente et al.[35], who investigated the behavioral consequences of concurrent high-dose manganese exposure and restraint stress in adult male rats. The results suggested that restraint stress and high manganese exposure might interact at neurotransmitter levels while producing opposing effects, indicating complex neurochemical interactions. Similarly, Linares et al.[36] examined how restraint stress modulated uranium-induced oxidative damage in brain tissues of adult male rats. Although restraint stress showed minimal additional adverse effects at the tested uranium doses, it was concluded that the potential influence of concurrent stress should not be underestimated.

On the other hand, the interactive effects of perfluorooctane sulfonate (PFOS) exposure and maternal restraint stress were also extensively studied in our laboratory[37][38][39][40]. Both PFOS and restraint stress induced maternal toxicity, while PFOS exposure increased prenatal mortality. Restraint stress enhanced fetal toxicity only at the lowest PFOS dose. Long-term behavioral assessments of offspring revealed complex interactions, with animals exposed to both PFOS and restraint stress showing reduced mobility and sex-specific learning impairments. Postnatal development studies demonstrated that PFOS and restraint stress produced opposing developmental effects.

The above studies collectively demonstrate that concurrent stressors can significantly modify chemical toxicity through multiple mechanisms, often in dose-dependent, sex-specific ways, producing both synergistic and antagonistic effects. Regarding this, Jonker et al.[41] reviewed studies on combined exposures to chemicals and non-chemical stressors (e.g., noise, heat, restraint), highlighting a significant knowledge gap. The lack of data on these interactions complicates risk assessments, as non-chemical stressors can modulate chemical toxicity, necessitating integrated approaches to evaluate cumulative health impacts.

Experiments on Mammals

Mammalian studies have provided crucial insights into mixture toxicity mechanisms and dose-response relationships under controlled conditions. Sánchez et al.[6] investigated the nephrotoxic effects of concurrent mercury (as mercuric chloride) and uranium (as uranyl acetate) exposure in adult male Wistar rats. Their findings unequivocally demonstrated that combined exposure resulted in significantly more severe kidney damage (functional impairment and structural lesions) than predicted by summing individual effects, strongly indicating a potent synergistic interaction. Regarding developmental toxicology, Bellés et al.[11] assessed the combined effects of lead, methylmercury, and arsenic in pregnant Swiss mice. Co-exposure led to notable alterations in the toxicokinetics of individual metals and exacerbated certain developmental toxicities, such as reduced fetal weight and increased skeletal anomalies, compared to single metal exposures at equivalent doses, strongly suggesting synergistic interactions. This research highlighted the importance of exposure timing and developmental stage in determining mixture effects. Furthermore, Bellés et al.[42][12] examined combined postnatal administration of cesium-137 and nicotine in developing Swiss mice, finding that combined exposure led to significant modifications in anxiety-like behavior and impaired learning and spatial memory compared to controls or singly exposed groups, suggesting synergistic or additive detrimental impacts.

The complexity of mixture interactions was also demonstrated by Heredia et al.[16], who investigated neurobehavioral effects of concurrent neonatal exposure to cesium-137 and paraquat in developing CD-1 mice. Co-exposed mice exhibited significantly more pronounced or qualitatively different alterations in anxiety-related behavior and spatial learning/memory compared to controls or singly exposed groups, suggesting synergistic or potentiated neurotoxic profiles. Interestingly, Heredia et al.[43] also showed that bisphenol A co-exposure could ameliorate radiation-induced learning impairments in mice exposed postnatally to cesium-137 and BPA, suggesting protective interactions under certain exposure scenarios.

Pesticide mixture research by Moser et al.[10] using N-methylcarbamate combinations (seven different carbamates) in preweaning and adult Sprague-Dawley rats revealed different interactive properties for different mixing ratios. The principle of dose addition (concentration addition) provided reasonably accurate prediction for cholinesterase inhibition. However, varying chemical proportions could lead to some deviations, highlighting that relative contribution and potency are critical factors. Relative potency mixtures showed dose additivity for most endpoints while environmental mixtures demonstrated greater-than-additive responses. The results of that study emphasized that mixing ratios significantly influence interaction patterns and highlighted the importance of environmentally relevant exposure scenarios in mixture toxicity assessment. On the other hand, Howdeshell et al.[44] reviewed antiandrogenic phthalate mixtures in rats, finding dose-additive suppression of fetal testosterone production. Phthalate mixtures with antiandrogenic pesticides also showed additive effects, supporting cumulative risk assessments. These findings, combined with human biomonitoring data, indicated that individual phthalate risk estimates might underestimate health impacts, necessitating CRA approaches. Subsequently, Conley et al.[45] studied PFOA and PFOS co-exposure in pregnant Sprague-Dawley rats from gestation day 8 to postnatal day 2. Combined exposure shifted PFOA dose-response curves toward effects at lower doses for shared endpoints, with dose addition accurately predicting most outcomes except for less-than-additive maternal weight gain. These results support cumulative effects and the use of dose-additive models for PFAS mixtures. A summary of key findings is shown in Table 1.

Study	Chemical Mixture	Key Findings
Sánchez et al. [6]	Mercury and Uranium	Combined exposure resulted in significantly more severe kidney damage than predicted by summing individual effects, indicating a potent synergistic interaction.
Bellés et al.[11]	Lead, Methylmercury, and Arsenic	Co-exposure led to notable alterations in the toxicokinetics of individual metals and exacerbated certain developmental toxicities, such as reduced fetal weight and increased skeletal anomalies.
Heredia et al. [16]	Cesium-137 and Paraquat	Co-exposed mice exhibited significantly more pronounced or qualitatively different alterations in anxiety-related behavior and spatial learning/memory compared to controls or singly exposed groups.
Moser et al. [10]	N-methylcarbamate combinations	Varying chemical proportions could lead to some deviations from dose addition, highlighting that relative contribution and potency are critical factors.
Howdeshell et al.[44]	Antiandrogenic phthalate mixtures	Dose-additive suppression of fetal testosterone production, supporting cumulative risk assessments.
Conley et al. [45]	PFOA and PFOS	Combined exposure shifted PFOA dose-response curves toward effects at lower doses for shared endpoints, with dose addition accurately predicting most outcomes except for less-than-additive maternal weight gain.

Table 1. Key findings from experimental animal studies

Experiments on Aquatic Organisms

The aquatic environment provides an ideal testing ground for mixture toxicity research due to controlled exposure conditions and well-established test organisms. Barata et al.[46] investigated lethal and sublethal responses of *Daphnia magna* to binary combinations of metals (cadmium, copper, zinc) and pyrethroid insecticides (cypermethrin, deltamethrin). Their results revealed a highly complex spectrum of interactions; notably, certain metal-pyrethroid mixtures exhibited clear synergism. Importantly, model predictive abilities changed across endpoints. The independent action concept accurately predicted mixture toxicities for lethal responses of dissimilarly acting chemicals, whereas concentration addition

was more appropriate for feeding responses, irrespective of chemical mode of action. This finding challenged traditional assumptions about the relationship between pharmacological mode of action and mixture prediction models. Extending that work, Barata et al.[47] examined joint toxicity effects on offspring production in *D. magna* using binary mixtures targeting different biological processes. Their results indicated that the dominant ecotoxicological rather than pharmacological mode of action should guide mixture effect predictions for life-history traits. In parallel, Syberg et al.[7] experimentally explored mixture toxicity using *D. magna* exposed to binary and ternary mixtures of potassium dichromate (metal), sodium dodecyl sulfate (anionic surfactant), and carbaryl (carbamate insecticide). The authors reported that both CA and IA models equally predicted binary and ternary mixtures of similar- and dissimilar-acting toxicants in *D. magna* immobilization experiments, highlighting the importance of endpoint selection and underscoring the need for considering the mode(s) of action when selecting predictive models. For mixtures with different modes of action, deviations from simple additivity were more frequently observed.

Further research by Barata et al.[48] using *Ceriodaphnia dubia* demonstrated that selection of mixture toxicity models based on ecotoxicological mode of action provided more accurate predictions than those based on pharmacological mode of action when assessing population growth rate responses. This finding reinforced the concept that biological organization level influences the most appropriate mixture assessment approach. In turn, Pavlaki et al.[49] tested the toxic effects of two neonicotinoid insecticides (imidacloprid and thiacloprid) and nickel chloride on *Daphnia magna*, examining reproduction, survival, and body length. Nickel chloride was the most toxic compound, followed by thiacloprid and imidacloprid when tested individually. The mixture of imidacloprid and thiacloprid showed synergistic effects on reproduction (neonate production) at sublethal doses, while body length effects followed a concentration addition model. The imidacloprid-nickel mixture displayed no interaction for reproduction, but showed dose-dependent effects on body length, synergistic at low doses and antagonistic at higher concentrations. More recently, Maloney et al.[50] assessed acute (96-h) toxicity of neonicotinoid mixtures (imidacloprid, clothianidin, thiamethoxam) in *Chironomus dilutus*. Binary and ternary mixtures showed dose-level and dose-ratio-dependent synergism, deviating from concentration-additive predictions, with LC50 values of 4.63–55.34 μg/L for individual compounds. These findings demonstrate significant departures from additive toxicity models and underscore the necessity for empirically-based revisions to current water quality criteria and regulatory frameworks. Maloney et al.[51] extended this to chronic (28-day) exposures in *C. dilutus,* finding dose-ratio-dependent synergism in imidacloprid-thiamethoxam

mixtures, with up to 10% greater emergence reduction and male-dominated sex-ratio shifts, emphasizing the need for further research on neonicotinoid mixture effects in aquatic ecosystems. Key findings are summarized in Table 2.

Study	Chemical Mixture	Key Findings
Barata et al.[46]	Metals (Cadmium, Copper, Zinc) and Pyrethroid Insecticides (Cypermethrin, Deltamethrin)	Certain metal-pyrethroid mixtures exhibited clear synergism, and model predictive abilities changed across endpoints.
Barata et al.[47]	Binary mixtures targeting different biological processes	The dominant ecotoxicological rather than pharmacological mode of action should guide mixture effect predictions for life-history traits.
Syberg et al.[7]	Potassium Dichromate, Sodium Dodecyl Sulfate, and Carbaryl	Both CA and IA models equally predicted binary and ternary mixtures of similar- and dissimilar-acting toxicants in *D. magna* immobilization experiments.
Pavlaki et al.[49]	Neonicotinoid insecticides and nickel combinations	The imidacloprid-nickel mixture displayed no interaction for reproduction but showed dose-dependent effects on body length; synergistic at low doses and antagonistic at higher concentrations.
Maloney et al.[50]	Neonicotinoid mixtures (Imidacloprid, Clothianidin, Thiamethoxam)	Binary and ternary mixtures showed dose-level and dose-ratio-dependent synergism, deviating from concentration-additive predictions.
Maloney et al.[51]	Imidacloprid and Thiamethoxam	Dose-ratio-dependent synergism in imidacloprid-thiamethoxam mixtures, with up to 10% greater emergence reduction and male-dominated sex-ratio shifts.

Table 2. Key findings from aquatic toxicology studies

Human Epidemiological Evidence

Human studies provide the most direct evidence of mixture effects on health outcomes, though they present unique methodological challenges. Huang et al.[52] investigated the effects of multiple heavy

metal exposures on liver function in mining area populations of China, employing Bayesian kernel machine regression. Their findings revealed that cumulative exposure to arsenic, lead, and cadmium was significantly negatively associated with liver function, with lead contributing most substantially. Animal studies confirmed that co-exposure could aggravate liver dysfunction compared to single-metal treatments. Chang et al.[21] extended that research to non-contaminated rural populations, finding that higher concentrations of metal mixtures were positively correlated with indicators of poor liver function, with lead again showing the strongest contribution. Their analyses demonstrated possible interactions between cadmium and other heavy metals in affecting liver biomarkers. These findings are consistent with and supported by numerous animal studies demonstrating synergistic or additive hepatotoxicity from metal mixtures, highlighting translational relevance. Similarly, Yin et al.[22] conducted longitudinal studies revealing synergistic effects between cadmium-chromium on liver enzymes and three-way antagonistic effects of manganese-lead-chromium on albumin levels, illustrating the complexity of multi-metal interactions in human populations. Also in China, renal toxicity studies by Yin et al. [53] demonstrated significant impacts of both individual and combined heavy metal exposures on renal biomarkers, with synergistic effects observed for multiple metal pairs and antagonistic three-way interactions. These findings provided valuable insights into mechanisms linking multiple metal exposures to organ-specific toxicity in human populations under real-world exposure conditions.

Iin a contemporary review, Kassotis and Phillips[23] discussed complexities of assessing "Complex Mixtures and Multiple Stressors," advocating for increased use of whole-mixture testing, bioassays, and effect-based monitoring. In turn, Sprinkle and Payne-Sturges[54] examined US EPA's historical constraints in addressing mixture toxicity, finding statutory and internal barriers limited progress. Despite evidence of synergistic effects in biocide formulations, regulatory focus remained on single chemicals, contrasting with industry's use of synergism for lethality. The National Institute of Environmental Health Sciences (NHANES) pursued more scientific investigation, highlighting the need for regulatory reform. Recently, Haruna and Obeng-Gyasi[55] analyzed NHANES 2017–2018 data, finding non-linear, non-additive effects of PFOA, PFOS, cadmium, mercury, and lead on chronic kidney disease. Cadmium and mercury showed strong associations, with U- and N-shaped exposure-response relationships, emphasizing the need for advanced statistical methods and public health interventions to mitigate cumulative PFAS-metal effects. In turn, Itoh et al.[56] studied prenatal PFAS exposure in 15,131 Japanese mothers, finding no significant association with developmental delays in 4-year-olds across

five domains. However, unmeasured PFAS and postnatal exposures remain concerns, warranting continued investigation into long-term developmental impacts.

Pesticide Mixture Assessment

The regulatory landscape for pesticide mixtures has evolved significantly since the Food Quality Protection Act of 1996 mandated consideration of cumulative effects from chemicals with common mechanisms of toxicity. Wilkinson et al.[3] critically evaluated various methods for combining exposures to estimate risks from common mechanism chemicals, including hazard index, toxicity equivalence factor, and combined margin of exposure procedures. Their analysis revealed that the point of departure index and margin of exposure approaches were preferable because they separate policy-driven and data-based uncertainty factors, making the assessment process more transparent. Chen et al.[5] proposed formal statistical procedures for estimating cumulative risk by fitting dose-response models under dose addition assumptions, providing a crucial conceptual framework that influenced subsequent animal mixture studies. Boobis et al.[19] provided comprehensive guidance for cumulative risk assessment of pesticide residues in food, reviewing methodologies used by regulatory agencies. Their review emphasized that the main concern arises from dose addition of compounds acting by the same mode of action, though synergy should be addressed case-by-case where biologically plausible. These authors highlighted ongoing efforts to refine assessment strategies and the need for more data on pesticide interactions at low, environmentally relevant levels. The tiered approach recommended by the European Food Safety Authority (EFSA) provides a framework for resource-efficient cumulative assessment while maintaining scientific rigor. Recently, Payne-Sturges et al.[57] proposed a cumulative risk assessment (CRA) approach for seven phthalates under TSCA, using integrative physiology and common adverse outcome algorithms. These authors reviewed US EPA guidance and peer-reviewed literature, recommending adjustments to hazard indices and margins of exposure to determine "unreasonable risk," thereby enhancing regulatory decision-making for phthalate mixtures.

Statistical Methods and Data Analysis

Robust statistical methods are crucial for evaluating toxicological interactions among mixture components. Gennings et al.[58] proposed a unifying statistical concept for assessing toxicological interactions by focusing on changes in the slope of dose-response curves for mixtures compared to individual components. A four-step approach to evaluate mixtures for consistency with dose addition and

to characterize deviations (synergism or antagonism) was developed. This concept aligns with traditional additivity models in statistical literature while providing a quantitative framework for interaction detection and characterization from animal studies. El-Masri[59] reviewed experimental and mathematical modeling methods for investigating toxicological interactions, emphasizing limitations of empirical methods such as isobolograms and response surface methodology for extrapolation beyond experimental data ranges. Mechanistically-based models such as physiologically-based pharmacokinetic/pharmacodynamic models were highlighted as superior approaches because they include explicit interaction mechanism descriptions related to target tissue levels, emphasizing the need for scientific support from expert panels and laboratory toxicologists. Recently, Hao et al.[60] compared 11 statistical methods for mixture analysis, using simulations and a Puerto Rico birth cohort to assess chemical mixtures (metals, PAHs, phthalates, phenols) and birth outcomes. No single method excelled universally; Super Learner improved risk stratification for cumulative effects. The study provides guidelines for selecting methods based on research goals, identifying gaps for future development.

Physiologically-based toxicokinetic (PBTK) modeling is a promising approach for predicting mixture toxicity. Recent advancements in software and computational power have improved the accuracy of these models. Bart et al.[61] have provided a comprehensive overview of the current state of the art in PBTK modeling for mixtures, highlighting the challenges and opportunities in this field.

Theoretical Considerations and Limitations

Several reviews have examined the theoretical foundations and practical limitations of mixture toxicity assessment. Borgert et al.[8] challenged the premise that dose-response characteristics can be predicted from individual component MoA, citing examples where such predictions failed. The authors argued that while MoA information is invaluable, its application for predicting mixture effects, especially for chemicals with dissimilar or unelucidated MoAs, remains challenging. They emphasized that detoxification pathways must be understood before extrapolating dose addition to concentrations below no observable effect levels of mixture components, advocating for data-driven assessments. In turn, Lambert and Lipscomb[62] identified the lack of consensus on toxic mode of action definitions as a major rate-limiting step in mixture risk assessment advancement. They emphasized that for chemicals acting via similar mechanisms, concentration (dose) addition is generally most appropriate, whereas for dissimilarly acting chemicals, independent action might be suitable but cautioned about real-world mixtures with diverse or interacting MoAs. These authors proposed that critical evaluation of data at all

biological organization levels for key event identification could facilitate appropriate mixture assessment approach selection. Teuschler[4], in a key guidance-oriented paper, discussed the complexities of deciding which chemical mixtures risk assessment methods work best, reviewing approaches from component-based methods to whole-mixture testing. Teuschler[4] highlighted the importance of considering data availability, similarity of chemical structures/MoAs, and regulatory context, advocating for a tiered or weight-of-evidence approach. The dose addition and isobole method limitations were examined by Bosgra et al.[63], who demonstrated that these approaches have restricted applicability and can result in incorrect interaction conclusions under certain circumstances. They clarified conditions for each model, emphasizing dose addition for strictly similarly acting substances. Their physiologically-based mathematical modeling showed that chemicals with zero interaction could be misclassified as interacting using traditional isobole methods. Similarly, Løkke et al.[9] reviewed tools and perspectives for assessing chemical mixtures and multiple stressors. These authors highlighted the potential of mechanistically-based models that consider uptake and toxicity as time-dependent processes and advocated for a more holistic, systems-based approach. MoA schemes (e.g., [30][29][28]) enable in silico classification of chemicals, enhancing mixture risk prioritization.

Neurotoxicity and Developmental Effects

Neurotoxicity represents a particularly sensitive endpoint for mixture effects assessment. Dórea[15] reviewed lead-containing neurotoxicant mixtures during early life, finding that lead-associated effects from prenatal exposure create continued burdens on measurable neurodevelopment. The review, drawing on animal studies and human data, revealed that mixture potency and exposure timing showed measurable impacts on neurodevelopment, affecting cognitive function, behavior, and brain structure, though net effects and reversibility would require further investigation. Multiple exposures in children with autism spectrum disorders and attention deficit hyperactivity disorders strongly suggested lead-associated effects within mixture contexts, reinforcing the vulnerability of the developing brain. Mercury interactions with co-occurring neurotoxic substances were also examined by Dórea[64], who found that risks associated with multiple neurotoxicants depended on exposure type, timing, combinations, and environmental or genetic factors. Fish-methylmercury exposure during pregnancy and lactation was frequently confounded by opposing effects of neuroactive nutrients, leading to variable neurobehavioral test outcomes. Dórea[65] also reviewed aluminum and mercury neurotoxic effects, drawing on human epidemiological studies and experimental animal models. Animal studies showed co-exposure can lead

to enhanced oxidative stress, neuroinflammation, and more severe neurobehavioral deficits. Dórea[65] noted that while both elements share neuro-pathogenic pathways, their combined effects in Thimerosal-containing vaccines represent the most widespread binary mixture exposure in developing countries.

Carcinogenicity and Low-Dose Effects

The potential for low-dose chemical mixtures to contribute to carcinogenesis was examined by Goodson et al.[1] in "The Halifax Project." The authors reviewed 85 chemicals across 11 hallmark cancer phenotypes. Only 15% showed evidence of dose-response thresholds, while 59% exerted low-dose effects, suggesting that cumulative effects of individual chemicals acting on different pathways could produce carcinogenic synergies. That analysis, synthesizing evidence from numerous sources including animal experiments, highlighted the need for basic research on low-dose mixture effects and revision of traditional mode of action frameworks, challenging single-chemical approaches to carcinogen testing. In turn, Hernández and Tsatsakis[2] emphasized that little was known about potential adverse effects from long-term exposure to complex mixtures at low doses near health-based reference values. They advocated for integrated approaches combining in vivo, in vitro, and in silico data with systematic reviews of high-quality epidemiological studies to improve mixture risk assessment robustness. Furthermore, Hernández et al.[66] updated knowledge on pesticide mixture interactions, synthesizing findings from numerous studies (many involving animal models). They noted that metabolic process interactions affecting biotransformation represented the most common synergism mechanism for pesticide combinations (e.g., organophosphates, carbamates, pyrethroids).

Systematic Review Findings

A comprehensive systematic review by Martin et al.[67] analyzed 1220 mixture experiments over a 10-year period (2009-2019), examining a large dataset of published mixture studies (including many animal experiments) to identify trends in non-additive interactions. The analysis revealed that approximately two-thirds of studies incorporated only two components and relied primarily on low-cost assays. Important toxicity outcomes relevant for human risk assessment were rarely addressed. Strikingly, relatively few claims of synergistic or antagonistic effects exceeded acceptable between-study variability boundaries. In fact, most observed mixture doses were within a two-fold range of predicted additive doses. However, synergistic and antagonistic interactions were frequently reported across various

chemical classes, biological systems, and endpoints. The review confirmed concerns about synergistic potential of triazine, azole, and pyrethroid pesticide combinations while identifying new evidence for endocrine disrupting chemicals and certain metal combinations, providing a valuable meta-perspective.

Discussion

The comprehensive body of evidence from experimental animal studies, as exemplified by the findings presented, converges on the definitive conclusion that the biological and toxicological consequences of concurrent chemical exposures are profoundly complex and frequently deviate from the simplistic assumption of straightforward additivity. This central tenet has been increasingly recognized and explored over decades of research[19][67]. While the principles of dose addition (or concentration addition) and independent action serve as essential theoretical anchors and default starting points[63][5], particularly for components believed to share a similar mode of action (MoA) as empirically supported in cases like carbamate studies[10] or for similarly acting toxicants in aquatic models[7], the substantial prevalence of observed synergistic interactions (e.g., mercury and uranium nephrotoxicity by Sánchez et al.[6], developmental toxicities by Colomina et al.[14] and Bellés et al.[11]), and antagonistic interactions consistently underscores the inherent limitations of relying solely on these idealized models, especially when dealing with dissimilar MoAs or complex biological feedback loops. The MoA of individual constituents is a critical determinant of the interaction profile[8][62]. Chemicals perturbing the same target are more predisposed to dose-additivity, yet toxicokinetic interactions (involving ADME alterations) can lead to non-additive outcomes, as demonstrated in metal mixtures[11]. Dissimilar MoAs increase the potential for unpredictable interactions.

Developmental stages (prenatal and early postnatal) represent periods of heightened vulnerability to chemical mixtures[11][42][12][13][14][15][65][16][43]. Notably, even low doses of individual components (below NOAELs) can collectively precipitate significant neurodevelopmental deficits or other adverse effects. Additionally, non-chemical stressors, such as maternal stress[13], can exacerbate these impacts.

Assessing low-dose, environmentally relevant mixtures is crucial[26][1]. Animal studies investigating such scenarios[42][12][43] are vital but demand meticulous design and sensitive endpoints. Importantly, relative proportions of components, not just absolute doses, influence outcomes[10].

Methodological advancements are fundamental to progress in this field. Physiologically-based toxicokinetic (PBTK) modeling[18] (Bart et al., 2002) can predict internal target tissue concentrations and

effectively account for kinetic interactions. Statistical approaches like the "unifying concept" by Gennings et al.[58] aid in characterizing interactions. Modern 'omics technologies and systems biology hold significant promise for identifying biomarkers and elucidating pathways, potentially leading to Adverse Outcome Pathways (AOPs) for mixtures[23][68][4][3].

Although there have been advances, major challenges remain. Because environmental mixtures are so complex, it is impossible to test every combination directly. As a result, scientists must use step-by-step testing methods and predictive models. Extrapolation from animal models to humans carries significant uncertainties, which are amplified for complex mixtures. Regulatory frameworks often rely on default assumptions (e.g., dose additivity) that may not always be sufficiently protective[2]. Encouragingly, growing human epidemiological evidence[21][22][53] linking real-world mixture exposures to adverse outcomes underscores the public health relevance and effectively complements mechanistic insights from animal studies.

Conclusions and Future Directions

The current review highlights the complexity of mixture toxicity, showing that traditional risk assessment models often fail to accurately predict the effects of chemical mixtures. Simple additive models are usually insufficient. While dose addition is a reasonable starting point, many mixtures behave differently due to factors like chemical composition and exposure conditions. Developing models that include toxicokinetic and toxicodynamic interactions is basic for improving the accuracy of mixture risk assessments. Concurrent exposure to multiple chemicals often leads to effects that are not the sum of individual toxicities, with synergism and antagonism being common. These interactions depend on various factors such as chemical identities, concentrations, modes of action, exposure timing, and organism characteristics. Early life stages are particularly vulnerable.

In this sense, future research should focus on creating standardized methodologies for mixture testing, studying environmentally relevant exposure scenarios, and investigating low-dose effects of complex mixtures. Combining epidemiological evidence with experimental data is essential for validating findings and informing regulations. Additionally, factors like poor nutrition, psychosocial stress, and climate change-related stressors need further investigation. Regulatory implications require assessment approaches to balance scientific rigor with practical resource constraints. Advanced technologies like in vitro high-throughput screening, alternative animal models, organ-on-a-chip technologies, and computational tools are relevant for prioritizing mixtures and reducing animal testing. More research is

also needed on temporal exposure dynamics, critical windows of susceptibility, and delayed-onset or transgenerational epigenetic effects. Assessment strategies should consider both chemical and biological factors to move beyond single-chemical paradigms.

In conclusion, sustained, innovative research and interdisciplinary collaboration are essential for developing robust regulatory tools to safeguard human and ecological health against the complex challenges of combined chemical exposures.

Statements and Declarations

Conflicts of interest

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Declarations

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