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Heartland Health Research Alliance Comments Submitted to EPA Docket: EPA-HQ-OPP-2024-0154

EPA's Proposed Re-Approval for 2026 of Over-the-Top Applications of Dicamba on GM Soybeans and Cotton

These comments are submitted by Dr. Thomas Green on behalf of the Heartland Health Research Alliance (HHRA), a non-profit organization conducting research on the impacts of farming systems on the environment and public-health (hh-ra.org). Dr. Green serves as chair of the HHRA Board, and founded the IPM Institute of North America. Co-authors include Dr. Asa Bradman, a professor of public health at the University of California, Merced, and is an expert in pesticide exposure assessments; Dr. Charles Benbrook is a former Executive Director of HHRA and expert in herbicide use, risk assessment, and regulation. The authors report no conflicts of interest related to this submission, and work on these comments was completed without compensation. Dr. Benbrook has served as an expert witness in pesticide litigation, including past and ongoing cases involving glyphosate-based herbicides and non-Hodgkin lymphoma.

HHRA's ongoing birth-cohort study -- Heartland Study -- is examining:

- The dramatic increase in the use of dicamba between 2017-2024 throughout the Midwest;
- Human exposures to dicamba, particularly pregnant women; and
- Potential reproductive and adverse developmental impacts resulting from prenatal and early-life exposures to dicamba and other pesticides.

To date, the Heartland Study has enrolled over 1,600 mother-infant pairs (MIPs). Initial enrollment occurs early in pregnancy. The HS protocol was published in 2023 (see https://bmcpublichealth.biomedcentral.com/articles/10.1186/s12889-023-17171-9). Urine samples are collected during each trimester from pregnant woman enrolled in the study; at least two samples have been secured from most woman enrolled to date. Sample collection protocols follow strict procedures consistent with NIH-funded studies.

The levels of 17-pesticide analytes are being quantified in all urine samples, including glyphosate and its primary metabolite AMPA, glufosinate and its metabolite 3-MPPA, dicamba, 2,4-D, and several insecticides. Measures of individual and collective pesticide exposures will be determined from concentrations in urine, and used to examine potential associations with adverse pregnancy and birth outcomes, and on children's health and development.

Given the serious agricultural and environmental impacts resulting from the approval and widespread use of dicamba on dicamba-tolerant soybeans in the Midwest since 2016, we do not understand why the pesticide industry, and the EPA, would consider registering new dicamba formulations labelled for over-the-top (OTT) applications. Herein we highlight several reasons why the EPA's Office of Pesticide Programs (OPP/EPA) should reconsider final approval of new dicamba OTT uses that are the subject of this rule-making.

New dicamba uses will increase exposures to the nearly 100 million Americans in and around the Midwest who will once again be breathing in dicamba for several months per year. Such inhalation exposures will begin anew around May, 2026 if EPA finalizes approval of the new OTT labels.

Volatilization and dicamba drift will directly expose hundreds of thousands of farmers, landscapers, plant breeders, and rural residents. Importantly dicamba-induced crop and landscape damage will harm businesses, resulting in economic and financial stress. Such episodes, individually and

collectively, will trigger tensions and litigation in rural communities, just as annual applications of OTT dicamba did from 2016 through the end of crop year 2024.

Three of the four-existing registrations for OTT dicamba applications were vacated by the 9th Circuit Court of Appeals in late 2024. The 9th Circuit acted because the EPA had "substantially understated risks that it [EPA] acknowledged and failed entirely to acknowledge other risks" (quote on page 3 of the July 22, 2025 updated EPA dicamba human health risk assessment).

Re-approval of essentially the same formulations and labels will almost certainly lead to another flush of lawsuits brought in local and state courts. Such cases will have to get in line behind an already significant backlog of personal injury and crop damage cases stemming from past herbicide use on GMO soybeans and cotton.

Moving forward with these OTT registrations will also assuredly lead to another review of the legality of the registrations by the 9th Circuit. For many reasons, including the serious concerns over adverse public health outcomes, the 9th Circuit may once again vacate the registrations because of the serious gaps and flaws in the EPA's dicamba human health risk assessment that are highlighted in these comments.

HHRA believes EPA must conduct a more thorough and data-driven assessment of recent and significant changes in dicamba exposure levels. A credible exposure assessment is a necessary first step in quantifying the reproductive and developmental impacts following exposures to dicamba.

Trends in Dicamba Use

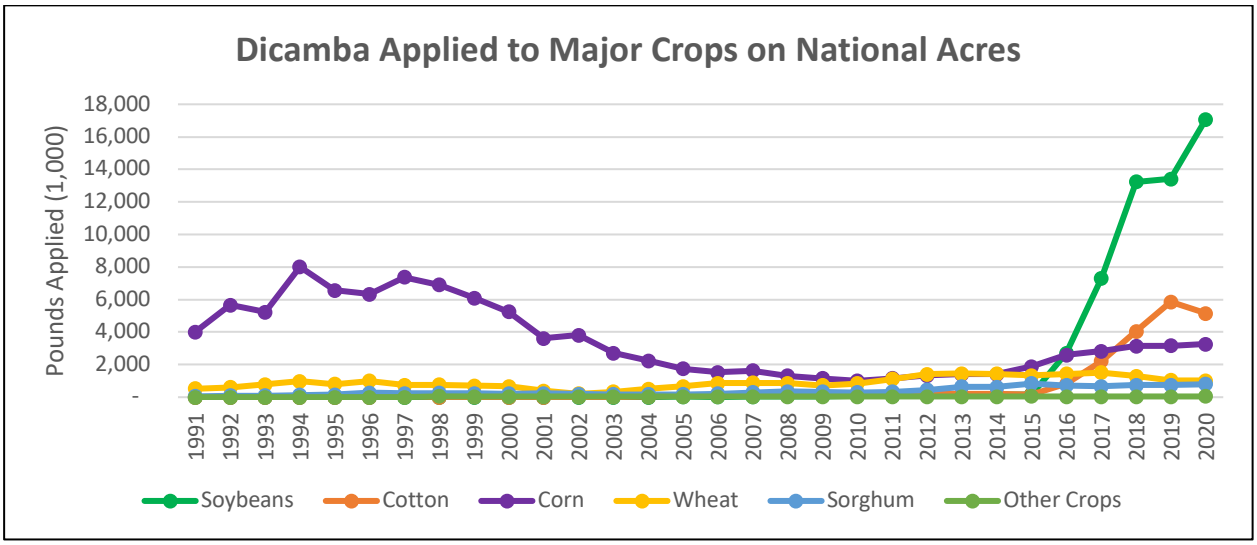
HHRA submitted comments dated October 17, 2022 to EPA on the known and likely consequences of OTT dicamba applications (access via the docket at regulations.gov: https://www.regulations.gov/comment/EPA-HQ-OPP-2016-0223-0691; or from the HHRA website at https://hh-ra.org/wp-content/uploads/2023/01/HHRA_Dicamba_Comments_10-17-22.pdf).

Several detailed tables reporting trends in dicamba applications on soybeans and cotton were included in the 2022 comments, and are referenced here as supporting information. The dicamba use data in these comments are reported as pounds of active ingredient applied. The tables and figures were generated from HHRA's Pesticide Use Data System (PUDS; access at https://hh-ra.org/projects/measuring-pesticide-use/pesticide-use-data-system/). PUDS draws upon the results of USDA pesticide use surveys as reported in QuickStats. PUDS utilizes the same methodology as the USDA's Economic Research Service in creating a continuous, annual data set of crop-specific information on pesticide use from periodic survey results.

Legal and rapid adoption of soybean and cotton seeds genetically engineered to tolerate OTT post-emergent applications of dicamba began in crop year 2017.[1] Through crop year 2024, dicamba-tolerant soybeans and cotton accounted for nearly three-quarters of dicamba crop use at the national level.

The dramatic and rapid impact of dicamba-tolerant seeds on the use of dicamba is a matter of record and is not in dispute. The figure below summarizes the trends in dicamba use by major crop since 1991 and through 2020 at the national level. Note that until GM dicamba-tolerant crops were approved, national dicamba use had fallen to trivial levels compared to most other herbicides.

[1] Several million acres of GMO, dicamba-tolerant seeds were planted in 2016, the year before EPA approvals were granted for dicamba-herbicide labels allowing post-emergent applications. Some of these planted acres were illegally sprayed with other dicamba formulations.



Prior to the major increase in dicamba use brought on by the planting of GMO dicamba-tolerant soybeans and cotton, dicamba use at the national level peaked at about 9.1 million pounds in 1994, and then declined steadily through 2010. The serious problems in rural areas caused by dicamba volatilization, off-field movement, and crop damage was a primary factor leading many farmers to avoid its use during this time period. The high levels of efficacy of Roundup Ready technology through the early 2000s also rendered unnecessary the use of most other herbicides on GMO corn, soybean, and cotton fields.

In the mid to late 2000s, row-crop farmers began spraying modest additional acres with dicamba prior to crop emergence, or post-harvest, in an effort to control weeds that had become resistant to glyphosate and other widely used herbicides. The rapid increase in dicamba pounds applied began in 2016 and reached 27.3 million pounds across all surveyed crops in 2020. Overall use rose over 11-fold from 2010 to 2020.

The table below presents dicamba use from 2021-2023 for all crops on which use was surveyed by the USDA. Peak national use from 1991-2023 occurred in 2021, when 27.6 million pounds were applied. Dicamba-tolerant soybeans and cotton accounted for 74.5% of reported national use that year, a proportion that fell marginally in 2022 and 2023.

The decline in dicamba use on cotton and soybeans between 2022 and 2023 was largely the result of growing supplies of Corteva's 2,4-D-tolerant soybean and cotton cultivars that competed directly with Bayer/Monsanto's Extend, dicamba-tolerant seeds.

Pounds of Dicamba Herbicide Applied to Crops Surveyed by the USDA in 2021 - 2023			
	2021	2022	2023
Barley	31,277	34,180	35,786
Corn	4,571,093	4,321,588	4,639,180
Cotton	4,340,787	5,321,340	3,959,365
Oats	8,364	7,746	6,950
Sorghum	1,036,653	897,581	-
Soybeans	16,205,742	14,615,435	12,447,649
Wheat, spring (excl. durum)	152,412	142,635	147,168
Wheat, spring durum	44,509	41,021	42,101
Wheat, winter	1,176,455	1,292,967	1,425,756
Totals	27,567,292	26,674,491	22,703,954
Two GM Dicamba-Tolerant Crops (Cotton, soybeans)	20,546,529	19,936,774	16,407,014
GM Crops as % Total Use	74.5%	74.7%	72.3%
Source: Pesticide Use Data System (PUDS), original data from USDA surveys and Quikstats. Calcualtions by Benbrook Consulting Services.			
Note: Values for crops not surveyed in a given year by USDA/NASS are interpolated between surveyed years.			

Since approval of dicamba-tolerant soybeans and cotton in 2017, ***dicamba use rose over 6-fold***, with dicamba-tolerant crops accounting for over 70% of national use. If EPA approves the three pending registrations sanctioning use of OTT dicamba products, dicamba pounds applied will increase, but likely less dramatically than between 2016 and 2021in light of the sizable market share now held by 2,4-D-tolerant seeds.

Significant Increases in Human Exposures to Dicamba in the Midwest Have Tracked the Planting of Dicamba-Tolerant Soybeans

In February, 2024, a Heartland Study (HS) team convened and supported by HHRA published the first, and to our knowledge, still the only dicamba human biomonitoring data collected since the approval of OTT dicamba applications on GM crops (open-access paper, Daggy et al., "Dicamba and 2,4-D in the Urine of Pregnant Women in the Midwest: Comparison of Two Cohorts (2010-2012 vs. 2020-2022)', *Agrochemicals* Vol 3; https://www.mdpi.com/2813-3145/3/1/5). The full text of this paper is included in Appendix A and is an integral part of this submission.

In 2020 and as part of the HS, HHRA commissioned the Center for Toxicological Research (CTQ)[2] in Quebec, Canada to develop a new and sensitive method capable of quantifying levels of dicamba and 2,4-D in human urine. CTQ was successful in doing so (download the open access methods paper at https://www.sciencedirect.com/science/article/pii/S004565352302619X). The new CTQ method has lowered the limit of detection by 5-fold to 10-fold for several of the 13 analytes detected. The availability of this method has also reduced the cost to test urine samples for both 2,4-D and dicamba about $200 per sample.

[2] CTQ in Canada (https://www.inspq.qc.ca/ctq/accueil) serves roles comparable to the US CDC in terms of pesticide analytical chemistry expertise and biomonitoring. It is a highly regarded lab internationally, and carries out multiple round-robin QA/QC tests in which labs around the world participate.

HHRA has access to stored, frozen urine samples collected in 2010-2012 from pregnant women residing in the Midwest and enrolled in the NIH-funded nuMoM2b birth-cohort study (NMM-study).[3] Samples were collected prior to the planting of dicamba-tolerant crops. Dicamba levels were quantified in 57 of these urine samples, along with levels of 2,4-D and metabolites of 10 pyrethroid and organophosphate insecticides.

In addition, HHRA submitted to CTQ for dicamba testing 86 samples of urine collected during 2020-2022 as part of the HS, after dicamba-tolerant soybeans had gained sizable market share in the Midwest, and the dramatic rise in dicamba use from 2016-2022.

In the 2010-2012 NMM-study samples, dicamba was detected in 16 of 57 (28%) of the samples (limit of detection (LOD) =0.1 ug/L). In the 2020-2022 HS samples, dicamba was present above the LOD in 60 of 86 (69.8%) of the samples. In summary, the frequency of samples with detectable dicamba rose 2.5-fold.

The increase in frequency of detection was more pronounced at the upper end of the distribution of dicamba concentrations, as evident in the changes in the number of samples above the higher Limit of Quantification (0.33 ug/L). In the NMM-study samples, 3 out of 57 (5.3%) were over the LOQ,. Among the HS samples, 39 of 86 (45.3 %) were over the LOQ. Thus, 8.5-fold more HS samples were over the LOQ, compared to the earlier NMM-study samples.

These changes in frequency between the 2010-2012 NMM-study samples and the 2020-2022 HS samples is unequivocal evidence of a major increase in exposure to dicamba in the Midwest. The only plausible source of this striking increase is the planting of dicamba-tolerant soybeans, and the concomitant increase in the pounds of dicamba applied on GM soybean crops. There were no other major changes in where and how dicamba was applied in the Midwest between 2010-2012 and 2020-2022. EPA has been aware of these facts for over three years, but has not addressed them in its 2025 updated dicamba human health risk assessment.

The distribution of dicamba levels in the 2010-2012 NMM-study samples compared to the 2020-2022 HS samples is shown in Table 3 below from the Daggy et al. paper. The differences in dicamba concentrations in urine are equally striking and worrisome.

Table 3. Distribution of concentration levels (μg/L) of dicamba in urine samples estimated assuming lognormal.

Cohort	N	25th %ile (95% CI)	Geometric Mean (95% CI)	75th %ile (95% CI)	95th %ile (95% CI)	*p*-Value
Not SG-standardized (assuming lognormal)						
nuMoM2b	57	0.020 (0.012, 0.034)	0.047 (0.030, 0.075)	0.113 (0.074, 0.172)	0.394 (0.250, 0.621)	
Heartland	86	0.098 (0.069, 0.139)	0.234 (0.175, 0.312)	0.556 (0.413, 0.750)	1.939 (1.271, 2.959)	
SG-standardized (assuming lognormal)						
nuMoM2b	57	0.029 (0.017, 0.048)	0.066 (0.042, 0.104)	0.153 (0.101, 0.231)	0.509 (0.326, 0.796)	<0.0001
Heartland	86	0.117 (0.084, 0.164)	0.271 (0.205, 0.358)	0.625 (0.468, 0.833)	2.081 (1.390, 3.116)	

Note: %ile = percentile; *p*-value obtained from SAS LIFEREG procedure comparing two cohorts on geometric mean while accounting for censoring of values below the LOD; SG: specific gravity. Note: the geometric mean of the lognormal distribution represents the 50th percentile.

[3] The NMM-study enrolled over 10,000 women from several regions in the US (see methods paper at https://pubmed.ncbi.nlm.nih.gov/25648779/). The HS team selected urine samples for testing associated with pregnant women enrolled in the Midwest.

The geometric mean level of dicamba in the NMM-study samples was 0.047 ug/L. In the 2020-2022 HS samples, the geometric mean was 0.234 ug/L, a concentration level 5.2-fold higher than in the early NMM-study samples.

At the upper 95th of the distribution of dicamba concentrations, 0.394 ug/L of dicamba was detected in the earlier NMM-study samples, and 1.939 ug/L in the HS samples, a 4.9-fold increase.

The EPA's Updated July, 2025 Dicamba Human Health Risk Assessment

The biomonitoring data published in 2023 by the HS team raise serious questions about the validity of the updated EPA dicamba human health risk assessment. According to the EPA, its most recent assessment is "robust" and "has not identified any human health or dietary risks" ("EPA Announces Proposed Decision to Approve Registration for New Uses of Dicamba, Outlines New Measures to Protect Human Health, Environment", EPA Pesticide Update, July 23, 2025; access at https://www.epa.gov/pesticides/epa-announces-proposed-decision-approve-registration-new-uses-dicamba-outlines-new).

In discussing "Next Steps" in its July 23 announcement, the EPA states that it "...will decide whether the registration action meets the standard for registration under the Federal Insecticide, Fungicide, and Rodenticide Act" (FIFRA). For reasons set forth below, HHRA is concerned that the EPA's updated dicamba human health risk assessment does not meet the requirements of either FIFRA, or the "reasonable certainty of no harm" standard set forth in the Food Quality Protection Act. It is probable that whether the evidentiary base analyzed by EPA meets statutory requirements will end up being reviewed and decided by the 9th Circuit.

The primary gap in the EPA's updated human health risk assessment is that it fails to address clear evidence of substantial increases in dicamba human exposures in the Midwest, and specifically, the approximate 5-fold increase in the frequency of detection of dicamba in the urine of pregnant women in the Midwest, and the over five-fold increase in the levels of dicamba detected in the 2010-2012 samples in contrast to the 2020-2022 samples.

In its July, 2025 updated dicamba risk assessment, the EPA offers no evidence pointing to an alternative route of exposure, or source of dicamba residues, that could account for such dramatic changes in the presence of dicamba in urine samples collected from pregnant women.

Moreover, the EPA was aware of the above changes in the presence of dicamba in the urine of pregnant women in the Midwest almost three years before the release of its July 22, 2025 dicamba human health risk assessment. The basic findings discussed above were first submitted to the EPA in October, 2022 comments submitted by HHRA to the EPA's dicamba docket as part of an earlier public comment period. The findings were reported in more detail in the peer-reviewed, open-access Daggy et al. paper in *Agrochemicals* published in early 2024, a paper that the EPA was no doubt aware of.

The dramatic increase in dicamba exposures in the Midwest surprised us, and should have also surprised the EPA. In addition, essentially all pregnant women, and other citizens in the Midwest, are exposed on many days to 2,4-D, given the fact that 2,4-D was detected in essentially 100% of HHRA samples.

Likewise, HS urine data confirm the findings of other biomonitoring studies in North America. CDC scientists reported the presence of glyphosate in 81 percent of ~2,000 urine samples tested as part of the 2011-2014 NHANES study (Ospina M, Schütze A, Morales-Agudelo P, Vidal M, Wong LY, Calafat AM. Exposure to glyphosate in the United States: Data from the 2013-2014 National Health

and Nutrition Examination Survey. *Environ Int*. 2022 Dec;170:107620. doi: 10.1016/j.envint.2022.107620. Epub 2022 Nov 4. PMID: 36368224; PMCID: PMC10240384).

In 2024, CDC presented unique data on the distribution of glyphosate levels in the general population and among male applicators of glyphosate-based herbicides (Chang VC, Ospina M, Xie S, Andreotti G, Parks CG, Liu D, Madrigal JM, Ward MH, Rothman N, Silverman DT, Sandler DP, Friesen MC, Beane Freeman LE, Calafat AM, Hofmann JN. Urinary biomonitoring of glyphosate exposure among male farmers and nonfarmers in the Biomarkers of Exposure and Effect in Agriculture (BEEA) study. *Environ Int*. 2024 May;187:108644. doi: 10.1016/j.envint.2024.108644. Epub 2024 Apr 11. PMID: 38636272). Over 90 percent of a group of farmers who had recently applied a GBH had glyphosate in their urine, and over 80 percent of samples collected from people with no known use or occupational exposure to a GBH (i.e., the general public) also contained glyphosate.

This paper reported two key, new insights into exposures of glyphosate. First, levels in urine peak very quickly (within hours) after an exposure episode, and decline sharply over 24 hours, and, second, applicators who wore gloves and other Personal Protective Equipment (PPE) had dramatically reduced exposure levels.

The first finding alerts applicators, the public health community, and the EPA that peak glyphosate exposure levels in people occur very soon after an exposure episode, and hence, unless a urine sample is taken soon after an exposure, the level of glyphosate detected in urine will significantly under-represent peak exposure levels. The second finding drives home the importance of commonsense PPE in reducing applicator exposure and risk levels, and in particular, chemical-resistant gloves. Both such findings are relevant in the case of estimating exposures to dicamba, yet have not been taken into account or addressed by the EPA.

A government-funded study published by Canadian scientists in 2022 tested the urine of about 2,000 pregnant women living across Canada (Ashley-Martin J, Huang R, MacPherson S, Brion O, Owen J, Gaudreau E, Bienvenu JF, Fisher M, Borghese MM, Bouchard MF, Lanphear B, Foster WG, Arbuckle TE. Urinary concentrations and determinants of glyphosate and glufosinate in pregnant Canadian participants in the MIREC study. *Environ Res*. 2023 Jan 15;217:114842. doi: 10.1016/j.envres.2022.114842. Epub 2022 Nov 18. PMID: 36410462).

The Canadian samples were collected in 2008-2011 during the first trimester of pregnancy, and tested for glyphosate, AMPA, glufosinate and its primary metabolite 3-MPPA. The urine samples were analyzed at CTQ using the same method relied on in the testing of NMM-study and HS urine samples.

Glyphosate was detected in 74 percent of the Canadian samples, and AMPA was present in 72 percent. The authors stated that diet “was a probable route of exposure” because the presence of glyphosate and AMPA in urine varied modestly between the herbicide spray season and during winter months. The biomonitoring data collected by the HS also provides strong evidence of remarkable consistency in glyphosate and AMPA levels in urine over the year, and in women living in cities, suburban areas, and in heavily farmed regions.

These data suggest that in recent years, at least two-thirds of the pregnant women in the Midwest have been exposed to dicamba and other herbicides on a regular basis, and at markedly higher levels as a result of the widespread planting of dicamba-tolerant soybeans. In fact, most people in the Midwest are exposed to a half-dozen other herbicides, for a total of 8-10 compounds, on a near-daily basis during much of the year. Typical mixtures of herbicides in the urine of people in the Midwest include 2,4-D and dicamba; glyphosate and AMPA; atrazine and other triazine analytes; acetochlor and s-metalochlor and their metabolites; and other herbicides and analytes that are less frequently, present.

HHRA will soon receive the urine testing results from CTQ for several hundred additional HS samples, including some samples collected in 2024. The results will provide insights into how the shift in soybean acres to DuoEnlist, 2,4-D-tolerant soybeans has altered the levels of 2,4-D in human urine, and also impacted the level of dicamba in urine. These new data will allow the HS team to produce more recent and sophisticated assessments of the exposure patterns and levels of four important herbicides in human urine in the Midwest (glyphosate/AMPA, glufosinate/3-MPPA, 2,4-D, dicamba). HHRA will provide the new results to the EPA as soon as possible.

Implications of Rising Dicamba Exposures

The July, 2025 EPA dicamba human-health risk assessment does not address the fact that dicamba exposures in the Midwest have risen dramatically. The assessment does not provide any data or analysis on the expected increased levels of measured dicamba levels, as evident in the urine of women enrolled in the HS.

The EPA did not require additional studies or data. Their review does not even acknowledge the need to understand how the widespread planting of dicamba-tolerant soybeans in the Midwest has altered dicamba human exposures and risks among pregnant women, infants, young children, and the general public.

The fact that newborns in the Midwest now begin life carrying residues of multiple herbicides obtained from their mothers is of concern to the HS team and HHRA. It should also trigger the need for EPA to conduct a more thorough dicamba exposure and human health risk assessment than reflected in the EPA's cursory July, 2025 updated analysis.

The 1996 Food Quality Protection Act (FQPA) directs the EPA to impose an added 10-fold safety factor when setting human exposure thresholds for any pesticide applied on food crops, including dicamba, when one or both of two circumstances apply:

1. When available toxicology data does not support an EPA finding that aggregate exposures will be "safe" for pregnant women, infants, and children, and other high-risk population cohorts. Such a determination must be supported by high-quality toxicology data that shows that there is a "reasonable certainty of no harm" arising from current exposure levels.
2. When the EPA lacks the necessary data to carry out a credible estimate of exposures, and hence risks.

Clearly, the second of these two 10-X FQPA triggers applies in the case of dicamba exposures. It is also doubtful that the first criterion has been met. The EPA does not, however, apply the FQPA 10-X added safety factor in the July, 2025 dicamba human health risk assessment.

This issue will be among the factors the 9th Circuit will weigh in deciding whether, as a matter of law, the July, 2025 dicamba risk assessment, and the three, newly proposed registrations supported by that assessment, are legal or must be vacated.

Our analyses of dicamba levels in the urine of pregnant women will provide some of the first, rigorous assessments of likely dicamba-exposure pathways. New questions will become more pressing if dicamba levels change only modestly between the herbicide spray season in rural areas and the non-spray season in urban areas. What might explain such an unexpected temporal and spatial pattern of exposure?

If dicamba levels are consistently higher during the herbicide spray season among pregnant women living in rural ***and*** urban areas, such data would point squarely toward dicamba volatilization and off-target movement as a primary driver of rising levels of dicamba human exposure. But questions will

persist over likely routes and levels of exposure: dietary, drinking water, inhalation, dermal, and house dust.

These and other information gaps will make it difficult for farmers, the pesticide industry, and the EPA to take appropriate actions to reduce exposures and risk. We are confident the HS will produce valuable insights within just the next 1-2 years on trends in dicamba exposure levels. In the next 3-5 years, sufficient data should be available to support more detailed exposure and risk assessments among pregnant women, infants, and children.

The fact that herbicide use patterns on soybean and corn farms in the Midwest are certain to remain intense and fluid for many years will create difficult challenges for EPA and public-health risk assessors. It will also make it all the more important to address such challenges with the benefit of credible data relevant to real-world human exposures and potential risk outcomes.

Appendix A: Full text of Daggy et al., “Dicamba and 2,4-D in the Urine of Pregnant Women in the Midwest: Comparison of Two Cohorts (2010-2012 vs. 2020-2022)”, *Agrochemicals*; https://www.mdpi.com/2813-3145/3/1/5.

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Article

Dicamba and 2,4-D in the Urine of Pregnant Women in the Midwest: Comparison of Two Cohorts (2010–2012 vs. 2020–2022)

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Abstract: Currently, there are no known human biomonitoring studies that concurrently examine biomarkers of dicamba and 2,4-D. We sought to compare biomarkers of exposure to herbicides in pregnant women residing in the US Midwest before and after the adoption of dicamba-tolerant soybean technology using urine specimens obtained in 2010–2012 from the Nulliparous Pregnancy Outcomes Study: Monitoring Mothers-to-be (N = 61) and in 2020–2022 from the Heartland Study (N = 91). Specific gravity-standardized concentration levels for each analyte were compared between the cohorts, assuming data are lognormal and specifying values below the LOD as left-censored. The proportion of pregnant individuals with dicamba detected above the LOD significantly increased from 28% (95% CI: 16%, 40%) in 2010–2012 to 70% (95% CI: 60%, 79%) in 2020–2022, and dicamba concentrations also significantly increased from 0.066 µg/L (95% CI: 0.042, 0.104) to 0.271 µg/L (95% CI: 0.205, 0.358). All pregnant individuals from both cohorts had 2,4-D detected. Though 2,4-D concentration levels increased, the difference was not significant (*p*-value = 0.226). Reliance on herbicides has drastically increased in the last ten years in the United States, and the results obtained in this study highlight the need to track exposure and impacts on adverse maternal and neonatal outcomes.

Keywords: pesticide; exposure; 2,4-dichlorophenoxyacetic acid; human biomonitoring

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1. Introduction

The commercial launch of genetically engineered, glyphosate-tolerant soybean and cotton varieties in 1996 and corn in 1998 initiated a transformation in weed management systems in the US. The so-called "Roundup Ready" (RR) system simplified herbicide-based weed management systems and was highly effective [1]. From 1991 to 2010, the percentage of soybean and corn acres treated with glyphosate-based herbicides in the Midwest went up 30-fold and 20-fold, respectively [2]. Widespread and repeated applications of glyphosate-based herbicides over time triggered the emergence and spread of multiple glyphosate-resistant weeds [3,4]. As glyphosate efficacy waned, additional herbicides were needed to target glyphosate-resistant phenotypes. By 2010, multiple glyphosate-resistant weeds had become an economic problem on many farms. The pesticide –seed industry responded by engineering soybean and cotton cultivars to tolerate post-emergence, "over the top" applications of additional herbicides that could be used in conjunction with glyphosate-based herbicides within the RR seed system [5].

The majority of soybean and cotton seeds sold in the US are now genetically engineered to tolerate combinations of glyphosate, glufosinate, dicamba, 2,4-D (2,4-dichlorophenoxyacetic

acid), and the "fop" chemical family of ACCase (acetyl-CoA carboxylase) inhibitor herbicides. As a result, reliance on dicamba and 2,4-D has risen 10-fold or more compared to 2010 [6]. To further demonstrate the increase in dicamba and 2,4-D, we present the estimated kilograms (in 100,000 s) of dicamba applied each year to surveyed US cropland for the years 2010 to 2022 obtained from the Pesticide Use Data System (PUDS) [7]. The system utilizes data issued by the USDA's National Agricultural Statistics Service via QuickStats [8]. Values for years lacking NASS survey results are interpolated using the methodology developed by the Economic Research Service to produce a continuous temporal dataset from periodic survey results. Of note, the year 2022 is a forecasted level. From Figure 1, the overall level of dicamba use (kilograms applied in one hundred thousands) in the US has increased for soybeans since 2015 and slightly increased for cotton and corn. Levels do not indicate an increase in wheat, sorghum, or other crops.

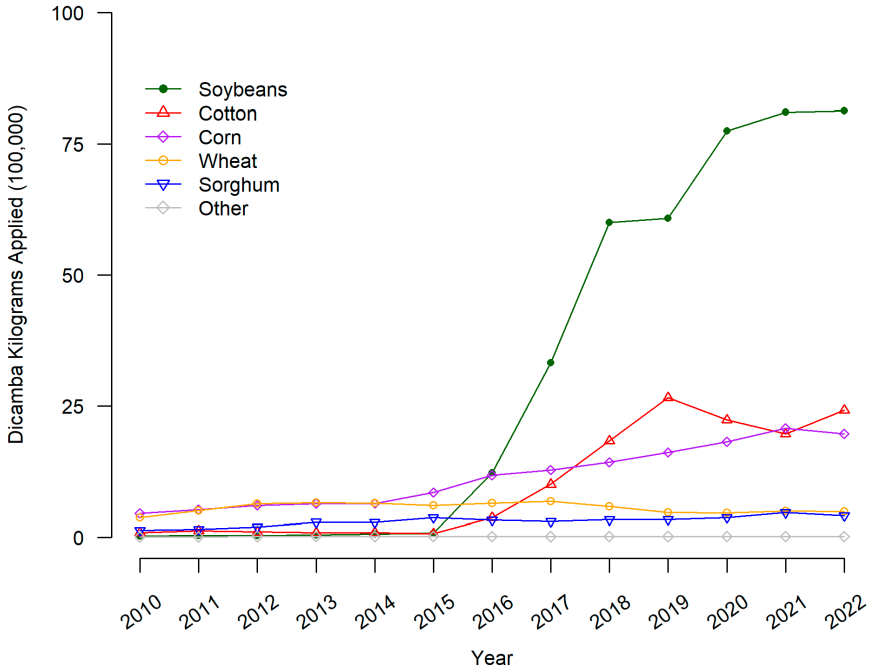

Figure 1. Dicamba applied to major crops on US surveyed hectares. Dicamba pounds applied is the sum of all forms of the herbicide reported in USDA-NASS surveys. Pounds applied was converted to kilograms by multiplying by 0.453592.

From Figure 2, the overall level of 2,4_D use (kilograms applied in one hundred thousands) in the US was highest in 2010 for wheat, soybeans, and corn. The amount of 2,4-D applied increased the most for soybeans and corn from 2010 to 2020 but remained relatively stable for wheat, sorghum, cotton, and other crops.

Similar figures of kilograms applied of dicamba and 2,4-D in 100,000 s of kgs to soybean crops are reported for the three midwestern states of the region where participants of the current study were enrolled (Illinois, Indiana, and Ohio).

Within the three midwestern states of interest, the kilograms of dicamba applied to soybean crops also has increased substantially (Figure 3). In Illinois, the amount of dicamba applied annually rose from approximately 2.9 kg (in one hundred thousands) in 2016 to over 9 kg (in one hundred thousands) by 2020, and 2,4-D increased also from 4.8 to 9.9 kg (in one hundred thousands) over the same time period. The increases in dicamba applied to soybean crops in Indiana and Ohio were also both observed but not as drastically as in Illinois. For example, in Indiana, the amount of dicamba applied annually rose from approximately 0.9 kg (in one hundred thousands) in 2016 to over 1.3 kg (in one hundred thousands) by 2020. The levels of 2,4-D applied to soybean crops within these midwestern states have varied with a continued increase in the amount of 2,4-D applied in Illinois

over the years and a marked increase in Indiana and Ohio in 2017, followed by a drop in 2018 and an increase thereafter. Across all three states, the amount of 2,4-D applied to soybeans has increased from 2010 to 2020, which is consistent with the US figure (Figure 2). As the use of genetically modified weed control methods increases, human exposure is also expected to increase [9]. If the increased use of dicamba and 2,4-D in the Midwest is reflected in the concentrations of analytes in the urine of pregnant individuals, this should result in a difference between the first cohort (2010–2012 prior to the initial use of dicamba-tolerant soybean technology) and the second cohort (2020–2022).

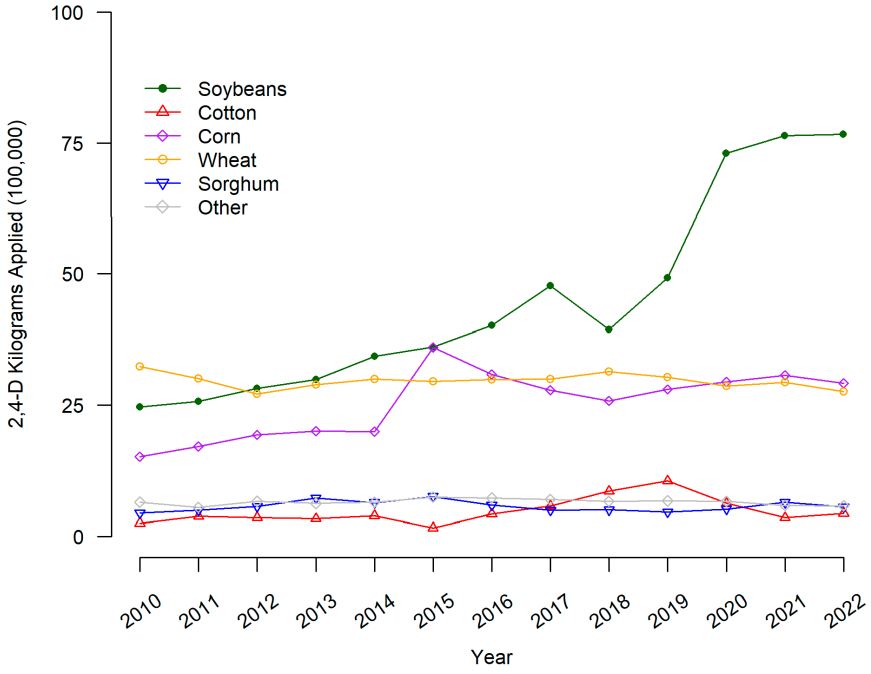

Figure 2. 2,4-D applied to major crops on US surveyed hectares. 2,4-D pounds applied is the sum of all forms of the herbicide reported in USDA-NASS surveys. Pounds applied was converted to kilograms by multiplying by 0.453592.

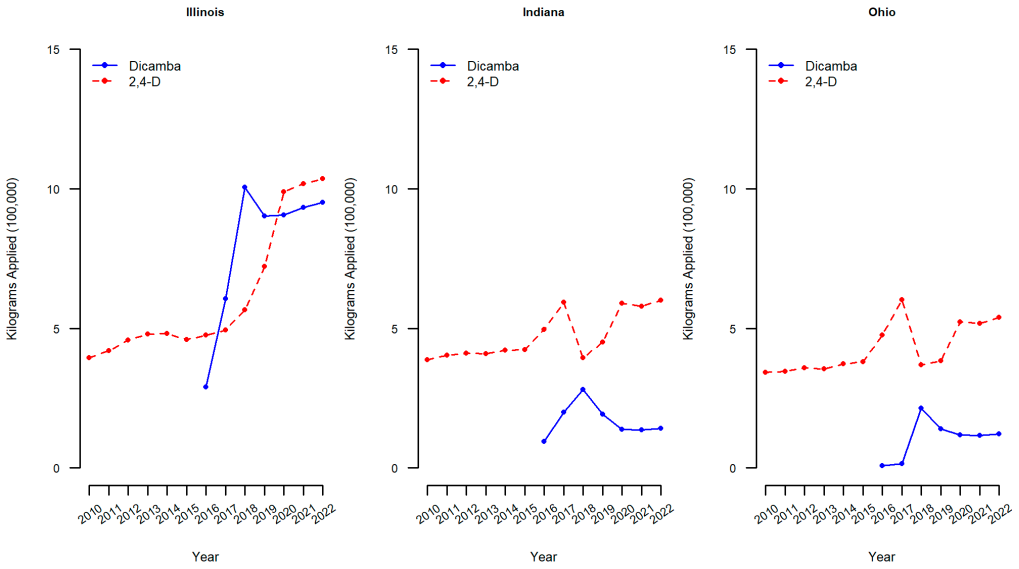

Figure 3. Dicamba and 2,4-D applied to soybean crops in midwestern states. Dicamba and 2,4-D pounds applied is the sum of all forms of herbicide reported in USDA-NASS surveys. Pounds applied was converted to kilograms by multiplying by 0.453592.

Although some studies have found associations between exposure levels of 2,4-D and adverse effects on maternal and/or neonatal outcomes, the results are limited. Urinary biomarkers of 2,4-D in 269 women were found to be associated with decreased head circumference [10], and biomarkers of 2,4-D measured in umbilical cord blood plasma in 232 women were found to be associated with deficits in auditory processing in infants [11]. However, another study of 858 mother–infant pairs found no association of 2,4-D measured in urine with outcomes of birth weight, gestational length, and abdominal circumfer- ence [12]. A more recent study measured levels of 2,4-D in urine collected longitudinally on 1225 pregnant women in China and found that approximately 97.4% of the urine samples contained 2,4-D and the levels were associated with biomarkers of oxidative stress [13]. The association of exposure to 2,4-D and biomarkers of oxidative stress has also been documented previously [14]. This is highly important since oxidative stress may be an underlying mechanism leading to adverse pregnancy outcomes [15].

Even fewer studies have explored the level of exposure to dicamba and the impact of exposure on adverse outcomes. Dicamba at low levels has similar hormonal properties to a class of plant hormones involved in cellular plant developmental processes [16], and at high concentrations, dicamba causes abnormal cell division and growth, disrupting normal plant functions, which results in death [17,18]. Previous studies that have examined levels of dicamba found in urine are relatively outdated and used assays that were not as accurate [19]; for example, only 1.4% of 400 urine samples obtained from a representative sample of the United States general population from 1976 to 1980 had quantifiable dicamba detected [20]. Our study is the first biomonitoring study with this level of accuracy to evaluate levels of dicamba in urine. Research is also still needed on the potential health effects of exposure to dicamba. From 3412 pregnancies from the Ontario farm study, exposure to dicamba 3 months prior to conception by self-report was found to be associated with an increased risk of birth defects in male offspring, although no association was found among all offspring [21]. Also, a case–control study of over 40,000 pesticide applicators found those in the highest quartile of exposure to dicamba had an increased risk of liver and intrahepatic bile duct cancer relative to those not exposed to dicamba [22]. However, no studies have examined the association of dicamba measured in urine with adverse pregnancy outcomes, hence the need for more research on the relationship between biomarkers of exposure and adverse effects on maternal and neonatal outcomes.

The Heartland Study (HS) was designed to fill this gap and is an ongoing Midwest-based birth cohort study that began in 2019. The goal of the study is to enroll at least 2000 mother–infant pairs to examine the association of prenatal herbicide exposure on pregnancy and childbirth outcomes as well as early childhood development [23]. The lack of a verified, sensitive, and cost-effective method to detect dicamba was identified as a key analytical gap. The fiscal sponsor of the HS, the Heartland Health Research Alliance (hhra.org), asked the Laboratoire du Centre de Toxicologie du Québec (CTQ) to develop a robust, selective, and sensitive method capable of detecting both 2,4-D and dicamba, along with other pesticide analytes, in urine, thereby producing a method suitable for biomonitoring in the general population. The new method was developed and validated by CTQ [19] and is coupled with CTQ's glyphosate-glufosinate method, which allows the HS team to quantify 17 pesticide analytes in urine samples.

The objective of this biomonitoring analysis was to assess whether biomarkers (concentration levels in urine) of dicamba and 2,4-D collected during pregnancy have increased in the Midwest (a geographic area where the use of these herbicides has increased substantially) by comparing a 2010–2012 cohort (i.e., nuMoM2b Study participants) to a 2020–2022 cohort (i.e., Heartland Study) using the newly validated 13-analyte method developed by CTQ. The method measures three analytes associated with herbicides (dicamba, 2,4-D, and 2,4-T), but 2,4-T is no longer registered for use in the United States; thus, dicamba and 2,4-D are the focus of the current analysis. Our results provide preliminary information on whether and to what degree 2,4-D and dicamba exposures have changed in the wake of the adoption of dicamba-tolerant soybean technology. Examining the association of maternal

herbicide exposure with adverse maternal and neonatal outcomes is beyond the scope of the present paper but is part of the overall goal of the ongoing Heartland Study [23].

2. Materials and Methods

2.1. Study Population

The HHRA accessed frozen urine samples collected in 2010–2012 as part of the NIH-funded (nuMoM2b) Nulliparous Pregnancy Outcomes Study: Monitoring Mothers-to-be project [24]. The full nuMoM2b study was an observational cohort from 8 study sites that enrolled 10,037 pregnant individuals. The current study used 61 samples collected in the first trimester from women enrolled in nuMoM2b from 3 of the study sites located in the Midwest as part of a smaller nested case–control study (Indiana University, Case Western University/Ohio State University, and Northwestern University). Specifically, cases were selected as participants in which any of the following occurred: hypertensive disorders of pregnancy, spontaneous preterm birth, gestational diabetes, stillbirth, or fetal demise < 20 weeks. Cases were matched to controls by participant characteristics such as age and smoking status. For the more recent cohort, samples of urine collected in 2020–2022 from pregnant individuals in their first trimester enrolled in the Heartland Study from Indiana were collected. Although the Heartland Study is ongoing, the earliest available 91 samples were analyzed for pesticide concentrations in the analysis.

The nuMoM2b study (ClinicalTrials.gov Identifier: NCT01322529) was approved by institutional review boards at all participating study sites. The urine specimens were obtained from a smaller substudy of this primary trial and received approval from the Indiana University Institutional Review Board on 24 May 2021 (protocol # 11666). The Heartland Study was approved by the Indiana University Institutional Review Board on 27 June 2019 (protocol # 1906366316).

2.2. Sample Collection

NuMoM2b urine samples were obtained during the first trimester from participants with a viable singleton gestation who were between 6 weeks + 0 days gestation and 13 weeks + 6 days gestation during an in-person study visit by trained research staff members. Samples were transferred to the laboratory for temporary storage until being sent to the central repository. Maternal urine specimens for Heartland participants were obtained during the first trimester (defined as up to 13 weeks + 6 days gestation) either during routine obstetrical visits by trained research staff members or self-collected remotely. Samples were allowed to be collected at any time of day. Frozen samples from both cohorts were shipped to Centre de Toxicologie du Québec (CTQ) for analysis.

2.3. Lab Methods

Urine specimens from both cohorts were analyzed using a validated method developed by the Centre de Toxicologie du Québec (CTQ) [19]. This method measures 13 biomarkers of pesticides exposure, including herbicides (dicamba, 2,4-D, and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)), organophosphates insecticides (malathion dicarboxylic acid (MDA), para-nitrophenol (PNP), 3,5,6-trichloro-2-pyridinol (TCPy), 2-diethylamino-6-methylpyrimidin-4-ol (DEAMPY), and 2-isopropyl-6-methyl-4-pyrimidinol (IMPY)), and synthetic pyrethroids insecticides (cis-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis-DCCA), trans-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (trans-DCCA), 3-Phenoxybenzoic acid (3-PBA), 4-Fluoro-3-phenoxybenzoic acid (4-F-3-PBA), and cis-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis-DBCA)).

Briefly, the metabolites in urine samples (250 µL) were hydrolyzed using 200 µL of a β-glucuronidase enzyme solution (6300 units/mL) in a pH 5.0 acetate buffer. After overnight incubation at 37 °C, a solid-phase extraction (SPE) of the pesticides' biomarkers was performed using Strata-X cartridges (30 mg/3 mL; Phenomenex, Torrance, CA, USA). The extracts were then analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring mode (MRM) using an Acquity I-Class UPLC

system (Waters, Milford, MA, USA) coupled to a Triple Quad 7500 System from AB Sciex (Concord, Ontario, Canada). The analytical column used was an Acquity Premier BEH C18 Column with VanGuard FIT (100 mm × 2.1 mm, 1.7 µm; Waters, Milford, MA, USA).

Specific gravity was measured using a refractometer.

2.4. Sample Size and Power

Prior to testing the hypothesis of whether there was an increase in pesticide concentrations in the more recent cohort, a power analysis was conducted. The number of available independent urine samples that had both dicamba and specific gravity measured was 57 in the nuMoM2b cohort and 86 in the Heartland Study cohort (Total N = 143). As the planned analysis was to compare the two cohorts on the specific gravity-adjusted log-transformed dicamba concentrations, power was based on a two-sample two-sided t-test, which provides 80% power to detect a standardized mean difference effect size (ES) of 0.48 at the 0.05 significance level, assuming the transformed measure is approximately normal. Because fewer urine samples were missing specific gravity for urine samples that also had 2,4-D concentrations, the available sample size for the 2,4-D outcome was N = 61 and N = 91 (Total N = 152), yielding 80% power to detect an ES = 0.47. As Cohen defines a medium effect size of ES = 0.50 [25], our hypotheses are appropriately powered to detect meaningful differences in concentration levels with the available sample sizes.

2.5. Statistical Methods

Machine-read herbicide analyte concentrations (µg/L) in the nuMoM2b and Heartland Study urine samples were provided by CTQ to the Heartland Study Data Coordinating and Analytics (DCA) core. The concentrations were normalized for dilution by a specific gravity adjustment using the formula Concentration × $[(SG_{reference} - 1)/(SG_i - 1)]$, where $SG_{reference}$ is a specified value derived from the cohort or a population value and SG_i is the specific gravity of the urine sample.

Potential ways to normalize for specific gravity include using the median (or mean) of either the cohort or some standard population-based cohort for reference. The most recent CDC-NIOSH manual references a specific gravity value of 1.020 as representative of the US population [26–28]. In the present analysis, we used a fixed value of 1.020 for the population value ($SG_{reference}$), which maintains consistency of standardization across cohorts and trimesters of pregnancy. Concentration values below the limit of detection were censored at the specific gravity-standardized limit of detection. Descriptive statistics for the specific gravity by cohort were also estimated.

To verify that the two cohorts represent a similar population of pregnant individuals, descriptive statistics for available demographic characteristics in both cohorts were provided. Spray season was defined as urine being collected between April and October. Although the exact date of sample collection was not available for nuMoM2b, most were collected at their Visit 1 assessment in the first trimester. Therefore, we assumed the collection date was the Visit 1 date. Quantiles (25th percentile, median, 75th percentile, 95th percentile) were estimated for dicamba and 2,4-D by cohort using machine-read values of concentrations that are not specific gravity (SG)-standardized, as well as SG-standardized values, assuming the data are lognormal.

For concentrations below the limit of detection (LOD), parameter estimates were obtained assuming data are lognormally distributed and specifying these values as left-censored [29]. To test our hypothesis that there is an increase in both analytes (dicamba; 2,4-D) in the most recent cohort (Heartland Study) compared to the historical cohort (nuMoM2b), which represents the pre-dicamba tolerant soybean period, the *p*-value is reported from the likelihood ratio test for comparing the two cohorts, assuming the data are lognormal. As spray season may influence exposure [13], the comparison of concentrations between cohorts was also conducted after adjusting for spray season to verify that the results were not affected.

The primary outcomes of interest are the specific gravity-adjusted concentration levels, although we also report the geometric mean and 95% confidence interval for the unadjusted concentration values. The proportion of participants (and 95% confidence intervals) with concentration values above the LOD and concentration values above the limit of quantification (LOQ) was estimated for each analyte by cohort. Additionally, empirical quantiles for dicamba, 2,4-D, and specific gravity are provided in the supplemental material with values below the limit of detection replaced with LOD/$\sqrt{2}$. The nuMoM2b samples were obtained as part of a nested case–control study from three study sites. Therefore, a sensitivity analysis was conducted where the primary comparison of SG-adjusted concentration levels between cohorts was re-estimated using inverse-probability weighting to account for the case–control status in the nuMoM2b participants. *p*-values < 0.05 were considered statistically significant. Statistical analyses were conducted in SAS Software, V9.4 (Cary, NC, USA).

3. Results

3.1. Descriptive Information of Pregnant Participants

For cohorts used in the dicamba comparison, most pregnant participants in this analysis were non-Hispanic with a mean age of 29, Table 1. A similar distribution of participants' race, ethnicity, education, and income was observed in both cohorts, although the mean gestational age at the first trimester was slightly higher in the nuMoM2b cohort (mean = 81.7 days, SD = 10.4 vs. mean = 73.3 days, SD = 15.5). Urine sample collection during the spray season was also comparable (68.4% of nuMoM2b samples and 60.5% of Heartland Health samples). Thus, the two cohorts are similar with respect to available demographic characteristics.

Table 1. Demographic characteristics of participants with urine samples included in dicamba comparison.

Characteristic	Overall N = 143	nuMoM2b N = 57	Heartland N = 86
Age, Mean ± SD	29.5 ± 5.6	29.3 ± 6.3	29.5 ± 5.1
Gestational Age (Days), Mean ± SD	76.6 ± 14.2	81.7 ± 10.4	73.3 ± 15.5
Maternal Race, N (%)			
- Black	29 (22.5%)	10 (17.5%)	19 (26.4%)
- White	80 (62.0%)	36 (63.2%)	44 (61.1%)
- Other	20 (15.5%)	11 (19.3%)	9 (12.5%)
- Missing, N	14		14
Maternal Ethnicity, N (%)			
- Hispanic	26 (20.2%)	10 (17.5%)	16 (22.2%)
- Non-Hispanic	103 (79.8%)	47 (82.5%)	56 (77.8%)
- Missing, N	14		14
Education, N (%)			
- Less than HS grad, HS grad, or GED	32 (26.7%)	11 (19.3%)	21 (33.3%)
- Some college or Assoc/Tech degree	25 (20.8%)	10 (17.5%)	15 (23.8%)
- Completed college	23 (19.2%)	12 (21.1%)	11 (17.5%)
- Degree work beyond college	40 (33.3%)	24 (42.1%)	16 (25.4%)
- Missing, N	23		23
Income, N (%)			
- USD 0–24,999	17 (16.0%)	6 (11.3%)	11 (20.8%)
- USD 25,000–49,999	16 (15.1%)	6 (11.3%)	10 (18.9%)
- USD 50,000–99,999	25 (23.6%)	12 (22.6%)	13 (24.5%)
- USD 100,000–149,000	21 (19.8%)	11 (20.8%)	10 (18.9%)
- USD 150,000–199,999	13 (12.3%)	10 (18.9%)	3 (5.7%)
- USD 200,000 or more	14 (13.2%)	8 (15.1%)	6 (11.3%)
- Missing, N Spray	37	4	33
Season, N (%)			
- Yes	91 (63.6%)	39 (68.4%)	52 (60.5%)
- No	52 (36.4%)	18 (31.6%)	34 (39.5%)

The nuMoM2b cohort for the dicamba comparison was obtained from participants enrolled in Illinois (64.9%, 37/57), Indiana (28.1%, 16/57), and Ohio (7%, 4/57). The Heartland cohort contained missing data for characteristics of race, ethnicity, education, and income ranging from 16% to 38% for the smaller cohort of 86 used in the dicamba comparison. As most of the participants for the comparison of 2,4-D concentrations overlap with the presented dicamba participants, the differences in demographic characteristics between the nuMoM2b and Heartland cohorts are similar, Appendix A (Table A1). A similar distribution by state was also observed for the nuMoM2b participants in which 2,4-D was measured: Illinois (63.9%, 39/61), Indiana (26.2%, 16/61), and Ohio (9.8%, 6/61), with all Heartland participants recruited from Indiana sites.

3.2. Dicamba and 2,4-D Measured in Urine

Dicamba was detected in urine samples at levels above the LOD (0.1 µg/L) for 28% (95% CI: 16%, 40%) of the nuMoM2b cohort and 70% (95% CI: 60%, 79%) of the Heartland Study cohort; therefore, the proportion of women with dicamba detected in their urine is significantly higher in the more recent cohort (Table 2). Concentration levels were detected above the LOQ (0.33 µg/L) in 5% (95% CI: 0%, 11%) and 45% (95% CI: 35%, 56%) of the nuMoM2b and Heartland Study cohorts, respectively, Table 2. Participant urine samples for 2,4-D concentrations were all above the LOD (0.01 µg/L) for both cohorts. Almost all, 99% (95% CI: 97%, 100%), of the 2,4-D concentrations from the Heartland Study cohort were above the LOQ (0.034 µg/L). The median specific gravity for the Heartland Study cohort was 1.020 for both dicamba and 2,4-D samples and was 1.017 for both dicamba and 2,4-D in the historical nuMoM2b cohort; that is, both cohorts had values very near the US population value we used as our reference (i.e., 1.020), Appendix A (Table A2).

Table 2. Proportions of concentration levels of dicamba and 2,4-D in urine samples above thresholds by cohort.

		Above LOD Values		Above LOQ Values	
Dicamba	N	N > LOD (0.1 µg/L)	Proportion (95% CI)	N > LOQ (0.33 µg/L)	Proportion (95% CI)
nuMoM2b	57	16	0.28 (0.16, 0.40)	3	0.05 (0.00, 0.11)
Heartland	86	60	0.70 (0.60, 0.79)	39	0.45 (0.35, 0.56)
2,4-D	N	N > LOD (0.01 µg/L)	Proportion (95% CI)	N > LOQ (0.034 µg/L)	0.05 (0.00, 0.11)
Heartland	61	61	1	61	1
nuMoM2b	91	91	1	90	0.99 (0.97, 1)

The specific gravity (SG)-standardized geometric mean of each for the two analyte concentrations is reported for the two cohorts, assuming data are approximately lognormal. For levels of dicamba concentrations below the LOD, parameter estimates were obtained, considering these values to be left-censored. A significant increase in dicamba concentration was observed in the most recent Heartland cohort as compared to the historical nuMoM2b cohort. The SG-standardized geometric mean of dicamba concentration was 0.066 µg/L (95% CI: 0.042, 0.104) in the nuMoM2b cohort and 0.271 µg/L (95% CI: 0.205, 0.358) in the Heartland Study cohort, with a statistically significant difference in dicamba concentrations (p-value < 0.001), Table 3. The difference in the SG-standardized geometric mean concentrations for 2,4-D between the two cohorts was not statistically different (p-value = 0.226); the 2,4-D concentrations were 0.383 µg/L (95% CI: 0.321, 0.458) in the nuMoM2b cohort and 0.442 µg/L (95% CI: 0.382, 0.511) in the Heartland Study cohort, Table 4.

Table 3. Distribution of concentration levels (µg/L) of dicamba in urine samples estimated assuming lognormal.

Cohort	N	25th %ile (95% CI)	Geometric Mean (95% CI)	75th %ile (95% CI)	95th %ile (95% CI)	*p*-Value
Not SG-standardized (assuming lognormal)						
nuMoM2b	57	0.020 (0.012, 0.034)	0.047 (0.030, 0.075)	0.113 (0.074, 0.172)	0.394 (0.250, 0.621)	
Heartland	86	0.098 (0.069, 0.139)	0.234 (0.175, 0.312)	0.556 (0.413, 0.750)	1.939 (1.271, 2.959)	
SG-standardized (assuming lognormal)						
nuMoM2b	57	0.029 (0.017, 0.048)	0.066 (0.042, 0.104)	0.153 (0.101, 0.231)	0.509 (0.326, 0.796)	<0.0001
Heartland	86	0.117 (0.084, 0.164)	0.271 (0.205, 0.358)	0.625 (0.468, 0.833)	2.081 (1.390, 3.116)	

Note: %ile = percentile; *p*-value obtained from SAS LIFEREG procedure comparing two cohorts on geometric mean while accounting for censoring of values below the LOD; SG: specific gravity. Note: the geometric mean of the lognormal distribution represents the 50th percentile.

Table 4. Distribution of concentration levels (µg/L) of 2,4-D in urine samples estimated assuming lognormal.

Cohort	N	25th %ile (95% CI)	Geometric Mean (95% CI)	75th %ile (95% CI)	95th %ile (95% CI)	*p*-Value
Not SG-standardized (assuming lognormal)						
nuMoM2b	61	0.150 (0.119, 0.188)	0.270 (0.217, 0.336)	0.487 (0.387, 0.612)	1.136 (0.865, 1.491)	
Heartland	91	0.213 (0.176, 0.257)	0.383 (0.320, 0.458)	0.690 (0.570, 0.836)	1.611 (1.265, 2.050)	
SG-standardized (assuming lognormal)						
nuMoM2b	61	0.238 (0.197, 0.286)	0.383 (0.321, 0.458)	0.619 (0.514, 0.745)	1.232 (0.988, 1.537)	0.226
Heartland	91	0.274 (0.234, 0.320)	0.442 (0.382, 0.511)	0.713 (0.611, 0.833)	1.421 (1.168, 1.729)	

Note: %ile = percentile; *p*-value obtained from SAS LIFEREG procedure comparing two cohorts on geometric mean while accounting for censoring of values below the LOD; SG: specific gravity. Note: the geometric mean of the lognormal distribution represents the 50th percentile.

As spray season could be a potential confounder, the models were also fit including cohort and spray season as explanatory variables. Spray season was not statistically significant in either the model for dicamba (p-value = 0.483) or 2,4-D (p-value = 0.998). The estimated geometric means and associated 95% confidence intervals by spray season and cohort are reported (Table 5). The results still hold in that the SG-standardized geometric mean of dicamba concentrations was still significantly higher in the more recent Heartland Study cohort (p-value < 0.0001) after adjusting for spray season. Similarly, the results for 2,4-D were also similar in that concentrations were not found to significantly differ by cohort even after adjusting for spray season (p-value = 0.227).

As the urine samples for nuMoM2b were part of a nested case–control study, a sensitivity analysis was conducted to verify the robustness of the results accounting for the study design. The SG-adjusted concentration values of the nuMoM2b cohort were reweighted to reflect the full cohort of women from the three study sites. The prevalence of cases (hypertensive disorders of pregnancy, spontaneous preterm birth, gestational diabetes, stillbirth, or fetal demise < 20 weeks) in the full nuMoM2b study cohort from the three study sites was 7.8% (207/2669), whereas the prevalence of cases for the nuMoM2b urine samples was higher (36.8% (21/57) and 37.7% (23/61) for dicamba and 2,4-D samples, respectively) due to the case–control design. After accounting for the case–control status of

the nuMoM2b study population by using inverse-probability weighting, the results were similar, Appendix A (Table A3). Specifically, dicamba concentrations were still significantly different ($p < 0.0001$) and 2,4-D concentrations were not statistically different ($p = 0.118$) between the two cohorts. The empirical quantiles (i.e., sample quantiles) for dicamba and 2,4-D are provided in Appendix A (Table A4) with values below the LOD replaced with LOD/$\sqrt{2}$.

Table 5. Concentration levels (µg/L) of dicamba and 2,4-D in urine samples by spray season and cohort estimated assuming lognormal.

Cohort	Spray Season	N	Concentration Levels (µg/L) Geometric Mean (95% CI)
SG-standardized dicamba (assuming lognormal)			
nuMoM2b	Yes	39	0.070 (0.044, 0.111)
	No	18	0.059 (0.033, 0.104)
Heartland	Yes	52	0.290 (0.207, 0.406)
	No	34	0.244 (0.164, 0.365)
SG-standardized 2,4-D (assuming lognormal)			
nuMoM2b	Yes	41	0.383 (0.316, 0.465)
	No	20	0.383 (0.302, 0.486)
Heartland	Yes	55	0.442 (0.372, 0.525)
	No	36	0.442 (0.361, 0.542)

4. Discussion

To our knowledge, no new dicamba human biomonitoring data have been generated since the substantial increase in dicamba use worldwide, brought about by the global launch in 2016 of dicamba-tolerant soybeans and cotton. The percent of pregnant women with detectable levels of dicamba in their urine in the 2020–2022 cohort is 2.5 times higher than in the earlier cohort (2010–2012), and a four-fold increase in mean concentration levels of dicamba was also observed over the same time period. This increase suggests the presence of a substantial new source of exposure.

In its most recent dicamba human health risk assessment, the US Environmental Protection Agency (EPA) [30] addressed the propensity of dicamba to volatilize and move with the wind. The pesticide manufacturers who won approval for dicamba formulations to be used "over the top" of soybeans and cotton invested heavily in the search for less volatile formulations, with some success. Based on the very limited field data quantifying dicamba movement from treated fields, the EPA concluded that "over the top" applications of dicamba on dicamba-tolerant soybean and cotton fields would not lead to more frequent or higher levels of human exposure to dicamba.

Residues of dicamba in food are minimal according to the United States Department of Agriculture's pesticide data program, which collects data on pesticide residues in multiple sampled food items [31]; thus, it is likely that a combination of inhalation exposure and the presence of dicamba in drinking water accounts for the changes in the frequency and levels of dicamba reported herein. As more urine samples are collected and tested by the Heartland Study team, we will explore the individual and joint impacts of characteristics such as place of residence (urban versus rural) and time of year (spray season, not spray season) on the frequency of detections and the distribution of concentration levels in urine. The results will help sharpen insights into the likely major sources of exposure to these herbicides in the midwestern US, a key step in mitigating exposures if any are deemed to be above acceptable risk thresholds.

Our work assessing the impacts of dicamba, 2,4-D, and other pesticide exposures on reproduction and children's development is ongoing. Securing accurate pesticide exposure metrics is a key challenge and step because the magnitude of changes in the last decade in herbicide use patterns and intensity in the Midwest is unprecedented. This is the reason the Heartland Study is collecting and storing ample urine and buccal swabs for each mother–infant pair—to allow additional, future research when, for example, data on

developmental outcomes are available and/or new tools to track markers of epigenetic impacts are developed.

The sequencing of DNA from infants born in the study and their parents is a particularly promising next step that will hopefully advance the identification and application of markers of genetic and epigenetic changes stemming from prenatal pesticide exposure. Within one or a few years of exposure, such markers can then be used to identify the presence of impacts known to be associated with neurodevelopmental problems or adult-onset disease. Such insights have the potential to markedly reduce the time required to link prenatal pesticide exposure to a heightened risk of adverse birth and health outcomes, thereby supporting regulatory interventions, when deemed necessary, a decade or more earlier than typically would be the case.

The EPA should continue monitoring newly published studies shedding light on herbicide-driven changes in reproductive outcomes and children's development and place more weight on published, high-quality studies. As described previously [32], greater reliance on published, peer-reviewed science can accelerate progress in pesticide risk assessment science. Revisiting the data accessible to the EPA to project the movement of phenoxy herbicides in the air should also be considered. Given that inhalation exposure is a likely cause of the substantial increase in exposure documented herein, a reassessment of dicamba volatility is needed to empirically evaluate the conclusion that there is no significant increase in exposure to dicamba as a result of the approval of dicamba-tolerant soybeans and cotton.

One limitation to note is that the Heartland Study participants were all obtained from study sites within Indiana, whereas the historical nuMoM2b participants were obtained from Indiana, Ohio, and Illinois; thus, the regions do not completely overlap. However, based on Figure 2, we would expect participants from Illinois to have the highest exposure levels, and these participants are only in the earlier cohort. Therefore, if Illinois participants had been in the more recent cohort, the levels of dicamba and 2,4-D may have been even higher in the more recent cohort. We did not limit the nuMoM2b analysis to Indiana due to sample size issues. Although participants were all enrolled in the Midwest region, due to the inability to obtain residential addresses for the nuMoM2b cohort, determining whether participants resided in an urban or rural setting was not feasible. Another limitation is that the time of day of urine collection was also not documented in either cohort.

In conclusion, using the newly validated method developed by the Laboratoire du Centre de Toxicologie du Québec to quantify dicamba and 2,4-D concentration levels in urine, we found that dicamba in pregnant study participants increased significantly in the more recent Midwest cohort from 2020 to 2022 relative to the earlier cohort from 2010 to 2012. Concentration levels of 2,4-D also increased in the more recent cohort, but the difference was not statistically significant. These results were maintained when further adjusting for whether the sample was obtained during spray season. Importantly, 100% of the pregnant study participants had 2,4-D detected in their urine in both the 2010–2012 cohort and the 2020–2022 cohort. This mimics what was recently found in China, with 98.6% of urine samples obtained in the first trimester from 1225 pregnant women in Wuhan, central China, containing 2,4-D, though concentration levels were higher in our US Midwest cohort (0.442 µg/L vs. 0.14 µg/L [ng/mL]) [13]. Although animal studies indicate negative health impacts on perinatal exposure to both 2,4-D and dicamba, human studies are still limited [33]. Epidemiologic studies have reported health impacts on adults, but the results are mixed. There is also a large amount of variability in how exposures have been measured (e.g., self-report, proximity to crops, urine, etc.). Additional research is still needed to elucidate the health implications of exposures to dicamba and 2,4-D on adverse pregnancy outcomes and the health of the offspring.

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Institutional Review Board Statement: The nuMoM2b study (ClinicalTrials.gov Identifier: NCT01322529) was approved by institutional review boards at all participating study sites. The urine specimens were obtained from a smaller substudy of this primary trial. The study was conducted in accordance with the Declaration of Helsinki and with approval from the Indiana University In- stitutional Review Board (protocol code 11666 and date of approval, 24 May 2021). The Heartland Study was conducted in accordance with the Declaration of Helsinki and approved by the Indiana University Institutional Review Board (protocol code 1906366316 and date of approval, 27 June 2019).

Informed Consent Statement: Written informed consent was obtained from all subjects in the Heartland Study. A waiver of authorization criteria was approved for the nuMoM2b study (protocol #11666) in accordance with 45 CFR 164.512(i).

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest: Charles Benbrook has served as an expert witness in litigation involving herbicides and was originally the Executive Director of the Heartland Health Research Alliance but stepped down to avoid any further conflicts of interest. He had no role in the analyses or interpretation of the data. No other author declares a conflict of interest.

Appendix A

Table A1. Demographic characteristics for participants with urine samples included in 2,4-D comparison.

Characteristic	Overall N = 152	nuMoM2b N = 61	Heartland N = 91
Age, Mean ± SD	29.3 ± 5.7	29.3 ± 6.6	29.3 ± 5.1
Gestational Age (Days), Mean ± SD	76.6 ± 14.1	81.7 ± 10.2	73.1 ± 15.4
Maternal Race, N (%)			
- Black	32 (23.4%)	12 (19.7%)	20 (26.3%)
- White	83 (60.6%)	36 (59.0%)	47 (61.8%)
- Other	22 (16.1%)	13 (21.3%)	9 (11.8%)
- Missing, N	15		15
Maternal Ethnicity, N (%)			
- Hispanic	27 (19.6%)	10 (16.4%)	17 (22.1%)
- Non-Hispanic	111 (80.4%)	51 (83.6%)	60 (77.9%)
- Missing, N	14		14
Education, N (%)			
- Less than HS grad, HS grad, or GED	34 (26.4%)	12 (19.7%)	22 (32.4%)
- Some college or Assoc/Tech degree	28 (21.7%)	11 (18.0%)	17 (25.0%)
- Completed college	25 (19.4%)	14 (23.0%)	11 (16.2%)
- Degree work beyond college	42 (32.6%)	24 (39.3%)	18 (26.5%)
- Missing, N	23		23
Income, N (%)			
- USD 0–24,999	17 (15.0%)	6 (10.9%)	11 (19.0%)
- USD 25,000–49,999	18 (15.9%)	6 (10.9%)	12 (20.7%)
- USD 50,000–99,999	27 (23.9%)	13 (23.6%)	14 (24.1%)
- USD 100,000–149,000	22 (19.5%)	11 (20.0%)	11 (19.0%)
- USD 150,000–199,999	14 (12.4%)	11 (20.0%)	3 (5.2%)
- USD 200,000 or more	15 (13.3%)	8 (14.5%)	7 (12.1%)
- Missing, N Spray	39	6	33
Season, N (%)			
- Yes	96 (63.2%)	41 (67.2%)	55 (60.4%)
- No	56 (36.8%)	20 (32.8%)	36 (39.6%)

Table A2. Distribution of specific gravity in urine samples from study cohorts.

Cohort	N	25th %ile	50th %ile	75th %ile	95th %ile
Specific gravity (dicamba samples)					
nuMoM2b	57	1.011	1.017	1.020	1.028
Heartland	86	1.012	1.020	1.025	1.030
Specific gravity (2,4-D samples)					
nuMoM2b	61	1.011	1.017	1.021	1.028
Heartland	91	1.012	1.020	1.025	1.030

Note: %ile = percentile.

Table A3. Distribution of concentration levels of dicamba and 2,4-D (µg/L) accounting for case– control status.

Cohort	N	25th %ile	50th %ile	75th %ile	95th %ile	Geometric Mean (95% CI)	*p*-value
SG-standardized dicamba (assuming lognormal, accounting for case–control status of nuMoM2b)							
nuMoM2b	57	0.032	0.074	0.169	0.562	0.074 (0.048, 0.114)	<0.0001
Heartland	86	0.118	0.271	0.624	2.071	0.271 (0.205, 0.358)	
SG-standardized 2,4-D (assuming lognormal, accounting for case–control status of nuMoM2b)							
nuMoM2b	61	0.228	0.368	0.593	1.181	0.368 (0.308, 0.440)	0.118
Heartland	91	0.274	0.442	0.713	1.419	0.442 (0.382, 0.511)	

Note: %ile = percentile; SG: specific gravity. To account for the case–control status of the nuMoM2b, cohort inverse-probability weighting was used to weight the nuMoM2b sample so that the sample reflects the prevalence of cases (hypertensive disorders of pregnancy, spontaneous preterm birth, gestational diabetes, stillbirth, or fetal demise < 20 weeks) in the full cohort. Weights for Heartland health were kept at 1.

Table A4. Empirical estimates of distribution of concentration levels of dicamba and 2,4-D (µg/L) by cohort.

Cohort	N	25th %ile	50th %ile	75th %ile	95th %ile
Not SG-standardized dicamba (values below LOD are substituted with LOD/$\sqrt{2}$)					
nuMoM2b	57	0.071	0.071	0.119	0.521
Heartland	86	0.071	0.285	0.601	1.879
SG-standardized dicamba (values below LOD are substituted with LOD/$\sqrt{2}$)					
nuMoM2b	57	0.079	0.129	0.236	0.553
Heartland	86	0.141	0.309	0.757	1.632
Not SG-standardized 2,4-D					
nuMoM2b	61	0.158	0.253	0.418	1.081
Heartland	91	0.206	0.426	0.641	1.665
SG-standardized 2,4-D					
nuMoM2b	61	0.246	0.351	0.526	1.970
Heartland	91	0.271	0.404	0.676	1.422

Note: %ile = percentile; SG: specific gravity; LOD: limit of detection.

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