Communication to City Council: GLYPHOSATE (G) 06082016

PREPARED FOR:
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City Commissioner: Steve Novick
City Commissioner: Amanda Fritz
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Auditor: Mary Hull-Caballero
Mayor: Charlie Hales

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June 8, 2016

PORTLAND PARKS & RECREATION
Healthy Parks, Healthy Portland

SUBJECT:

Logical Rationale for the Elimination of the Spraying of all Glyphosate Based Herbicides, in Parks Managed by Portland Parks & Recreation

RECOMMENDED ACTION:

For City Council to amend Portland City Council policy, pertaining to Oregon Statutes (ORS 262.1), Chapter 943, by updating the Integrated Pest Management (IPM) to discontinue the use of all currently City of Portland approved herbicidal products containing Glyphosate (Ranger Pro, RoundUp Concentrate, RU ProDry, Rodeo, Aquaneat, Aquamaster). This action is primarily directed at the over 7,000 acres natural areas managed by Portland Parks & Recreation. If this action is deemed as an acceptable and prudent measure, this provision should apply to the entire 11,500 acres currently under the jurisdiction of Portland Parks & Recreation.

FISCAL IMPACT:

Provided upon request, during a future scheduled meeting enabling a 10 minute communication.

STRATEGIC PLAN:

Provided upon request, during a future scheduled meeting enabling a 10 minute communication.

Glyphosate is a non-selective systemic herbicide. It penetrates and encompasses the entire plant that it is infecting, and will eradicate any additional plants that are not genetically engineered to resist it. Glyphosate's chemical effect is primarily to block enzymes that plants necessitate to exist, and it also reduces their production of amino acids and vital proteins. (Hoagland et al.) Glyphosate was patented by the Monsanto Company under the trade name *"RoundUp"* in 1973. There are currently over *"750 products"* containing glyphosate for sale in the U.S. (NPIC) In 1985, acting out on the scientific discoveries of tumor formations on mice, the EPA originally classified glyphosate as *"possibly carcinogenic to humans"*, placing this chemical into *"Group C"*. Six years later, the EPA oddly decided to alter its classification of glyphosate by moving it to *"Group E"*, declaring that it is *"non-carcinogenic to humans."* (IARC)

Five years later, in 1996, GMO resistant crops were introduced extensively into the U.S. agricultural sector by the Monsanto Corporation. Today, the EPA allows *"50 times more glyphosate"* for agricultural use than in 1996. (Main) Glyphosate possesses the *"highest global production of all herbicides"*. The U.S. *"consumes 25% of the world's supply of glyphosate"*, despite possessing less than 5% of the world's population. (Seneff) The agricultural use of this product has increased exponentially alongside the introduction of genetically modified crops, which are scientifically formulated to resist the negative effects caused by this toxic man-made organic substance. An astounding *"50%"* of American farmers fields now display weeds that actually have become resistant to glyphosate. (Main) The presence of glyphosate has been detected in the *"air during* (the actual) *spraying, in groundwater, and also in food"*, including non-GMO crops, all across the globe. (WHO)

The USDA analyzed glyphosate (G) residues in US soy in 2011, and surprisingly discovered that 271/300, *"90.3%"* of the samples provided tested positive for glyphosate, and 287/300, *"95.7%"* tested positive for AMPA, which is a byproduct of glyphosate breakdown cycle, which is equally as toxic. AMPA is a compound that is a specific adversary for the AMPA receptor, where it *"mimics the effects of the neurotransmitter glutamate"*. (Purves et al.) In the over 100 listed residues of pesticides listed by the USDA, only 11 other toxins were detected, in a combined 2.1% of the time. (USDA)

An abundance of studies that demonstrate *"low toxicity"* regarding this herbicide, are solely based on the active ingredient, glyphosate, and not the other inert ingredients in the formulation. RoundUp is *"41% glyphosate, and 59% inert ingredients"* (UMCP) These adjuvants, mixtures, which are considered inert by the manufacturer, that are protected under proprietary laws, as *"trade secrets"*, have been scientifically confirmed to *"amplify up to 1000 times the toxicity of their active principles, in 100% of the cases, where they are indicated to be present by the manufacturer*." (Defarge et al.) All glyphosate formulations are far more toxic than when tested in isolation, and possess the ability to penetrate all *"three human cell lines"* more significantly. (Bernay et al., 2012; Benachour et al., 2009; Richard et al., 2005)

The role of the adjuvants *"is to increase AP solubility, and to protect it from degradation, increasing its half-life"*, which aids cell penetration, thus *"enhancing its pesticidal activity"*. (Defarge et al.) Glyphosate has been scientifically demonstrated to affect human placental cell viability at subagricultural doses (0.1%), and alter sexual biosynthesis at lower nontoxic doses (0.01%). This was due to the *"highly amplified adjuvants"*, the *"so-called inert ingredients"* of RoundUp formulations. (Benachour et al.)

A study performed by the Bureau of Reproductive and Child Health, which is located in Ottawa, Canada, observed an associated risk linked to glyphosate that produced spontaneous abortions in an Ontario farm population. Their results revealed that "*among older women exposed to glyphosate, the risk for spontaneous abortion was three times higher than women of the same age who were not exposed to this active ingredient."* (Arbuckle et al.) A growing number of international experts in toxicology also consider the genotoxicity of glyphosate to be associated in producing higher risks of obtaining childhood brain cancer. (Bentz et al.)

During 2014, an international Advisory Group containing senior scientists and government officials, advised the World Health Organization to evaluate dozens of pesticides, to determine if there is any potential association with glyphosate, that could produce adverse life-threatening human health effects.

On March 20, 2015 the International Agency for Research on Cancer, which is part of the World Health Organization, that represents 194 member states internationally, declared glyphosate as a *"Group 2A"* carcinogen. This category is defined as *"probably carcinogenic to humans"*. Their justification for this classification was based on *"convincing evidence that glyphosate caused cancer in laboratory animals"*, and by evaluating significant findings from EPA reports, concluding that *"there is sufficient evidence of carcinogenicity"* that has been clearly documented during mammalian laboratory studies. The IARC Working Group also observed *"DNA and chromosomal damage* (occurring) *in human cells"* during this unbiased scientific research.

A study conducted in late 2010 in Berlin, Germany, found traces of glyphosate visible in human urine. The samples were provided by *"city workers, journalists, and lawyers, who had no direct contact with glyphosate"*. They were examined for contamination by a research team at the University of Leipzig, and all of the subjects tested positive. These were values ranging from *"0.5 to 2 ng glyphosate per ml urine (drinking water limit: 0.1 ng / ml)"*. (Brandli et al.)

Residues of glyphosate are found in a majority of *"foods of (contained in) the Western diet"*. Which are comprised primarily of corn, soy, wheat, and sugar. The *"negative impact on the body is insidious, and manifests slowly over time as inflammation damages cellular systems throughout the body"*. Glyphosate's negative interference relating to CYP (cytochrome P450) enzymes, restricts a critical role of human cellular biology. Glyphosate also leads to the disruption of beneficial gut bacteria, and produces the chelation of vital minerals and nutrients such as iron, cobalt, manganese, folate, zinc, vitamin K, vitamin D, among others. The chelation capacity of glyphosate has been proven to occur intracellularly in relationship to plant cells, and in animals, due to the adjuvants, which accelerate and intensify cell penetration. (Kruger et al. 2013; Hoppe et. al 2014) Glyphosate also disrupts neurotransmitters, depletes serotonin, melatonin, and dopamine. (Samsel et al.) It also inhibits the pituitary release of thyroid stimulating hormones, which leads to hypothyroidism. (Beecham et al.) International experts of toxicology have demonstrated glyphosate's ability to impede the progression of *"puberty, body development, the hormonal production of testosterone, estradiol, and corticosterone."* Glyphosate has also been scientifically proven to significantly alter testicular morphology. (Bernardi et al.)

After conducting extensive research for over a decade, Denmark scientists discovered that glyphosate can be filtered through various soil types, by the action of raining, which eventually sends these toxic herbicides into drains, leading into rivers, and then into oceans. (Brusch et al.) This particular study led to Denmark enacting a ban on glyphosate being used on paved surfaces, because of subsequent *"urban runoff"* poisoning their waterways. (Franzen et al.)

The IARC & WHO *"Group 2A"* classification lists numerous carcinogens that are extremely hazardous to humans. Some of these substances are even illegal, nationally, and internationally. *"Lead compounds (inorganic), petroleum refining, polybrominated biphenyls (PBB's), human papillomavirus (HPV), Dichlorodiphenyltrichloroethane (DDT), and Androgenic (anabolic) steroids"* are all listed in the same carcinogenic category as glyphosate.

The International Agency for Research on Cancer recommendations are based on quote *"scientific evidence based on a comprehensive view of the scientific literature"* end quote. Unfortunately, the implementation of enforcing these ethical standards are the responsibility of individual governments, and

other international organizations, who must establish the regulations, or legislation to act upon. The California Environmental Protection Agency's Office of the Environmental Health Hazard Assessment, published a notice on September 4, 2015, announcing its intent to list glyphosate, as known to the state, to cause cancer under the Safe Water & Toxic Enforcement Act of 1986, commonly known as Proposition 65. (OEHHA)

The chair of the federal House of Representatives Committee on Science, Space, & Technology has recently launched an investigation as of May 4, 2016. This is regarding the EPA's immensely anticipated report relating to potential negative human health effects, specifically pertaining to glyphosate. The EPA released a *"Final Report"* from the EPA's Cancer Assessment Review Committee, that was signed by 13 scientists, for the House Committee to review, and then the EPA immediately removed the data from the system. The House Committee on Science, Space, & Technology chair Lamar Smith, is demanding that the EPA produce every document and linked scientific study, and all communications records related to glyphosate, that transpired from January 1, 2015 to present time, to the committee. The deadline for the EPA to provide this critical data was May 18, 2016. The EPA has still not responded. The House Committee on Science, Space, and Technology has complete *"jurisdiction over (all) environmental and scientific programs"* and oversees the EPA. (Smith)

These vastly necessary EPA documents are being reviewed to determine if glyphosates can be considered as a safe practice in light of recent scientific research. This is the first time that the EPA *"fully analyzed the threats posed by glyphosate"* since *"1993"*. (Center for Biological Diversity) A growing number of member states located in the EU are seriously debating whether or not to discontinue glyphosate use after July of this year. A recent survey conducted across the five largest states, show immense support for the elimination of glyphosate entirely, by enacting a ban. Two-thirds of the total respondents were in favor of the ban. Italy demonstrated this highest support with *"75%"*, and the United Kingdom was the lowest at *"56%"*. (Neslen)

Full opt-out requests have been enacted by *"Austria, Bulgaria, Croatia, Cyprus, Denmark, France, Greece, Hungary, Italy, Latvia, Lithuania, Luxembourg, Malta, the Netherlands, Poland, and Slovenia"*. (Seneff) Sri Lanka, a country in Asia that has twice the population of Oregon and Washington combined, has seen fatalities related to chronic kidney disease (CKDu) increase by 500%, after the introduction of the herbicide Roundup, being applied to their farmlands two decades ago. Last year their president officially banned the importation of any substances containing glyphosate into their country. (Sri Lanka) El Salvador also banned glyphosate due to experiencing a similar epidemic of chronic kidney disease, as a byproduct of glyphosate implementation. Chronic kidney disease is surprisingly the *"second (leading) cause of death, among poor males"* in both farming countries. (SOR 2016; Jayasumana et al. 2014; CAD 2013; Ramirez-Rubio et al. 2013)

Our primary obstacle in providing adequate protection for residents living in the Portland metropolitan area, and all across the nation, is that we are relying on the EPA as our sole source for regulating this extremely toxic herbicide. The EPA represents a country with only 4.4% of the world's population, the World Health Organization represents 194/196 nation-states. On May 6, 2016, the Monsanto Company produced a peculiar statement on their twitter account. It was regarding this highly meaningful and

suspiciously unrevealed EPA study, which stated *"Have you heard glyphosate causes cancer? The EPA disagrees."* (Monsanto Company)

Why does the Monsanto Company have any access to this highly confidential information, that the House Committee on Space, Science, and Technology, who provides oversight to the EPA, cannot even obtain? The Monsanto Company has historically developed numerous highly toxic manmade organic chemicals, from PCB's, to DDT, that were banned for use in the 1970's, during the time period when glyphosate was actually formulated. (Monsanto Company) They have proven over and over again in the international community, that their primary interest is regarding economic gain, rather than protecting public health.

In 1948, the Universal Declaration of Human Rights was adopted and proclaimed as international law, by the UN General Assembly, by a unanimous decision. (Worldmark) Along with the International Covenant on Civil and Political Rights, these two international standards are primarily *"referred to as (part of) the international bill of rights"*. According to Article 3 of UDHR, and Article 9 of the CCPR, essentially, all inhabitants of the Earth are granted the equal right to possessing "*bodily integrity*", and maintaining the optimum security of protecting one's personal health. (Kerns 2014; UN 1976; UN 1948)

Unbiased, and non-industry funded credible science, must be the determining factor when making decisions to protect the health and security of humankind. This is how the World Health Organization made their logical and ethical decision on glyphosates, so why can't we be rational thinking human beings too?

Thank you sincerely for spending the time to read this. It is genuinely appreciated.

For any additional information pertaining to this testimony, or to any of the related intergovernmental scientific studies, please contact:

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Works Cited:

Arbuckle, Tye, Zhiqiu Lin, Leslie S. Mery. ***"An Exploratory Analysis of the Effect of Pesticide Exposure on the Risk of Spontaneous Abortion in an Ontario Farm Population"***. Bureau of Reproductive and Child Health, Population and Public Health Branch, Health Canada, Ottawa, Ontario, Canada; Cancer Bureau, Population and Public Health, Health Canada, Ottawa, Ontario, Canada. Environmental Health Perspectives Vol. 109:8. August 2001.

Benachour, Nora, Gilles-Eric Seralini. ***"Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells"***. American Chemical Society. Chemical Research in Toxicology. 22, 97-105. 2009.

Bentz, BJ, RC Brownson, JR Davis, R Garcia, A Turner. ***"A Family Pesticide use and Childhood Brain Cancer"***. Arch Environmental Contamination Toxicology 24:87-92. 1993.

Bernardi, MM, PV Furtado, CA Oliveira, MA Romano, RM Romano. ***"Prepubertal Exposure to Commercial Formulation of the Herbicide Glyphosate Alters Testosterone Levels and Testicular Morphology"***. National Center for Biotechnology Information. U.S. National Library of Medicine. Arch Toxicology 84(4):309-17. 12 Dec 2009.

Bernay, B, R Mesnage, G E Seralini. ***"Ethoxylated Adjuvants of Glyphosate-Based Herbicides are Active Principles of Human Cell Toxicity"***. University of Caen, Institute of Biology, France. U.S. National Library of Medicine. Journal of Toxicology. 10 Sept 2012.

Beecham JE, S Seneff. ***"Is there a Link Between Autism and Glyphosate-Formulated Herbicides?"***. Journal of Autism; ISSN 2054-992X; 3:1. 2016.

Brandli, Dirk, Sandra Reinacher. ***"Herbicides Found in Human Urine"***. Delinat Institute for Ecology and Climate-Farming. Viticulture Ecology Climate Farming. Ithaka Journal. Germany. Jan. 2012.

Brusch, W, V Ernstsen, L Gudmundsson, R Grant, RK Juhler, F Plauborg, P Nyegaard, P Olsen. ***"The Danish Pesticide Leaching Assessment Programme Monitoring Results May 1999-June 2009"***. Geological Survey of Denmark and Greenland, Ministry of Climate and Energy and Faculty of Agriculture Sciences. 2010.

Central American Database (CAD). ***"El Salvador: Use of 53 Chemicals Banned"***. 2008-2016 CentralAmericanData.com., Business Information. 6 Sept. 2013.

Center for Biological Diversity. ***"EPA Uses Industry-Funded Studies to Determine Glyphosate Does Not Cause Cancer"***. Portland, Oregon. 2 May 2016.

Defarge, Nicolas, Robin Mesnage, Gilles-Eric Seralini, Joel Spiroux de Vendomois. ***"Major Pesticides are More Toxic to Human Cells Than Their Declared Active Principles"***. BioMed Research International. Volume 2014, Article IS 179691. 26 Feb 2014.

Franzen, I, AC Grundy, C Kempenaar, P Kristoffersen, J Raisio, H Schroeder, A Verschwele, L Zarina. ***"A Review of Pesticide Policies and Regulations for Urban Amenity Areas in Seven European Countries"***. Weed Research 48(3). 2008.

Hopp, HW, M Kruger, W Lutz, P Schledorn, W Schodl. ***"Detection of Glyphosate Residues in Animals and Humans"***. Institute of Bacteriology and Mycology of Veterinary Faculty, University of Leipzig, Germany. Wildlife Research Institute, Bonn, Germany. Avian and Rabbit Diseases Department, Faculty of Veterinary Medicine, Sadat City University, Egypt. Journal of Environmental & Analytical Toxicology. 2014;4:210.

Jayasumana C, Gunatilake S, Senanayake P. ***"Glyphosate, Hard Water and Nephrotoxic Metals: Are they the Culprits Behind the Epidemic of Chronic Kidney Disease of Unknown Etiology in Sri Lanka?"***. U.S. Library of Medicine. National Institutes of Health. International Journal of Environmental Research and Public Health. 2014 Feb 20;11(2):2125-47

Kerns, Tom. ***"A Human Rights Impact Assessment of Auckland Transport's Road Corridor Vegetation Control"***. Environment and Human Rights Advisory. November 2014.

Kruger M, W Schodl, J Neuhaus, A Shehata. ***"Field Investigations of Glyphosate in Urine of Danish Dairy Cows"***. Journal of Environmental & Analytical Toxicology. 2013;3186.

Main, Douglas. ***"Glyphosate Now the Most-Used Agricultural Chemical Ever"***. Newsweek, Tech & Science. 2 Feb. 2016.

Monsanto Company. ***"Have you Heard Glyphosate Causes Cancer? The EPA Disagrees"***. monsanto.info/26MNNB3. Twitter.com @MonsantoCo. 2 May 2016.

---. ***"PCB's (Polychlorinated Biphenyl)"***. 2002-2015 Monsanto Company Website. 31 May 2016.

National Pesticide Information Center (NPIC). ***"Glyphosate General Fact Sheet"***. U.S. EPA, Oregon State University. 2016.

Neslen, Arthur. ***"Two-Thirds of Europeans Support Ban on Glyphosate, Says Yougov Poll"***. The Guardian. 11 April 2016.

Office of Environmental Health Hazard. ***"California Environmental Protection Agency Office of Environmental Health Hazard Assessment: Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65) - Notice of Extension of the Public Comment Period for Notice of Intent to List: Parathion, Malathion, and Glyphosate"***. State of California. 25 Sept. 2015.

Portland Parks & Recreation. ***"Integrated Pest Management Program: Includes Updates to 7/1/2015"***. City of Portland. 2016.

Purves, Dale, George J. Augustine, David Fitzpatrick, William C. Hall, Anthony-Samuel LaMantia, James O. McNamara, and Leonard E. White. ***"AMPA"*** Neuroscience. 4th Edition. Sinauer Associates. 2008.

Ramirez-Rubio, Oriana, Michael D McClean, Juan Jose Amador, Daniel R Brooks. ***"An Epidemic of Chronic Kidney Disease in Central America: An Overview"***. J Epidemiol Community Health. 2013;67.1-3.

Richard, S, S Moslemi, H Sipahutar, N Benachour, G E Seralini. ***"Differential Effects of Glyphosate and RoundUp on Human Placental Cells and Aromatase"***. Environmental Health Perspective. 113, 716-720.

Samsel A, Seneff S. ***"Glyphosate's Suppression of Cytochrome P450 Enzymes and Amino Acid Biosynthesis by the Gut Microbiome: Pathways to Modern Diseases"***. Entropy. 2013; 15(4):1416-1463.

---.***"Glyphosate, Pathways to Modern Diseases III: Manganese, Neurological Diseases, and Associated Pathologies"***. U.S. National Library of Medicine. Surgical Neurological International. 24 March 2015.

Seneff, Stephanie. ***"Impact of Glyphosate: Possible Modes of Action"***. CSAIL, Massachusetts Institute of Technology (MIT). Presentation in California. 6 March 2016.

Smith, Lamar. ***"The Honorable Gina McCarthy: Administrator - U.S. Environmental Protection Agency"***. Congress of the United States, House of Representatives. Committee on Science, Space, and Technology. Washington, D.C. 4 May 2016.

Sri Lanka. ***"Sri Lanka President Orders to Ban Import of Glyphosate with Immediate Effect"***. Sri Lanka Internet Newspaper. International Day for Biological Diversity at the Ministry of Mahaweli Development and Environment at Battaramulla. Colombopage.com. 22 May 2015.

Seattle Organic Restaurants (SOR). ***"Sri Lanka joins El Salvador in Banning Herbicide Roundup Linked to a Wide Range of Diseases Including Fatal Chronic Kidney Disease"***. Seattleorganicrestaurants.com. 2016.

United Nations (UN). ***"International Covenant on Civil and Political Rights"***. United Nations Human Rights, Human Rights Committee. Article 3. 23 March 1976.

---. ***"Universal Declaration of Human Rights"***. United Nations Human Rights, Human Rights Committee. Article 9. 10 Dec. 1948.

United States Department of Agriculture (USDA). ***"Pesticide Data Program Annual Summary, Calendar Year 2011"***. Agricultural Marketing Service. Science & Technology Programs. Appendix C. February 2013.

University of Maryland (UMCP). ***"Vinegar: An Alternative to Glyphosate?"***. University of Maryland, College of Agriculture and Natural Resources. 2014.

World Health Organization (WHO). ***"IARC Monographs Volume 112: Evaluation of Five Organophosphate Insecticides and Herbicides"***. International Agency for Research on Cancer (IARC). Lyon, France. 20 March 2015.

Worldmark. ***"United Nations"***. Encyclopedia of the Nations. Tenth Edition. ANSI X39, 478-1984. 2001.

Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells

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We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup (R) formulations, from 10^5 times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation. This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

Introduction

Humans are exposed daily to a great number of xenobiotics and their metabolites, present as pollutants (*1*). They act as mixtures having compensatory, multiplicative, or synergistic effects, as we have shown (*2*) with others (*3, 4*). The main glyphosate (G) formulations, commercialized as Roundup (R) from the Monsanto Company, are themselves already mixtures of G and various adjuvants at different concentrations. We have studied these products, which are the major nonselective herbicides worldwide (*5*); moreover, their use and presence in the food chain (*6*) are increasing since more than 75% of genetically modified edible plants have been designed to tolerate high levels of these compounds (*7*). G and its major metabolite aminomethylphosphonic acid (AMPA) were classified among the first contaminants in rivers (*8*). The adjuvants, less measured in the environment, are usually considered as inert and are protected as a "trade secret" in manufacturing (*9*). However, among them, the predominant one appears to be the polyethoxylated tallowamine or POEA (*10, 11*), which has itself some toxicity (*12*), such as causing ocular burns, redness, swellings and blisters, short-term nausea, and diarrhea. In combination with G, the mixture becomes more active (*13*). These products, like detergents, could allow facilitated G penetration through plasmatic membranes, potentialization of its action, increased stability, and bioaccumulation (*14, 15*).

The dose- and time-dependent cytotoxicity of R Bioforce (360 g/L of G, R360) on human placental and embryonic cells (*15*) could explain at least in part some reproductive problems (*16*). Among the two lines, 293 embryonic cells have proven to be very suitable for estimating the hormonal activity for xenobiotics (*17*), and JEG3 cells are also considered a useful model for examining placental toxicity (*18*). These lines may be equally or even less sensitive to xenobiotics than primary cultures (*19*). In the present study, we also tested the mechanism by which R mixtures affect human primary cells of the umbilical vein cord endothelial cells (HUVEC) for comparative purposes.

The endothelial lining of blood vessels constitutes a permeable barrier between the blood and the underlying tissues. The endothelium also plays an important role in various physiological processes, such as metabolism of vasoactive substances and maintenance of antithrombotic factors on the vessel wall (*20*). The endothelial cells are exposed directly to chemicals circulating in the blood of the umbilical cord and pass through the placenta (*21*). It is known that HUVEC cells may be a target for adverse effects of xenobiotics activated into reactive metabolites (*22, 23*). Other somatic cell types have been used to study pesticide toxicity and apoptosis such as HeLa (*24*) and Jurkat (*25*), but none was before treated by glyphosate.

In human cells, we have demonstrated that G mixed with adjuvants in R360 was cytotoxic through alteration of succinate dehydrogenase SD (*14, 15*). With isolated rat liver mitochondria, it is demonstrated that R depresses the mitochondrial complexes

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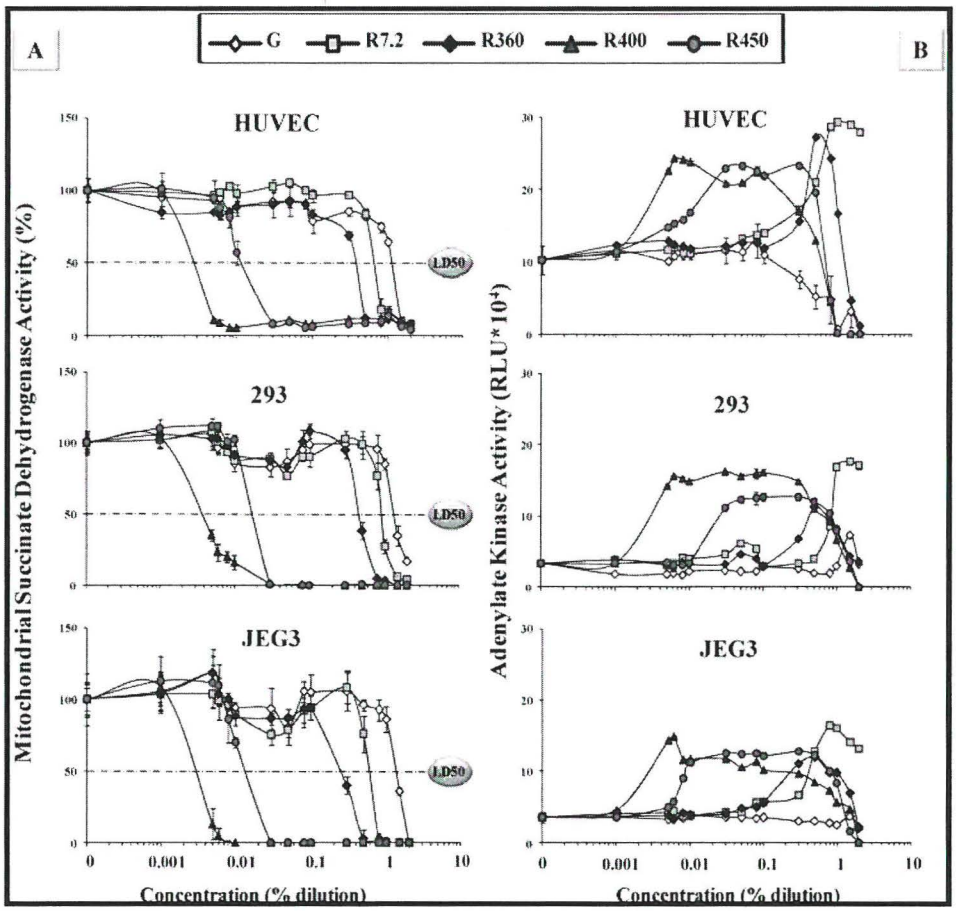

Figure 1. Cytotoxic effects of four Roundup formulations (R) on three human cell types. The R (from 10 to 2 × 10^4 ppm) contain different glyphosate (G) concentrations (7.2, 360, 400, or 450 g/L) and adjuvants. G alone was used as control at equivalent quantities to R360 and at similar pH 5.8. The cells were either primary from neonate umbilical cord (HUVEC) or lines from embryo (293) or placenta (JEG3). The actions on the mitochondrial succinate dehydrogenase (SD) activity (cellular viability in %, A) and on the release of cytoplasmic adenylate kinase (AK) activity [cell death in relative luminescence units (RLU), B] were compared in serum-free medium after 24 h of exposure. The 50% lethal dose (LD_{50}) is indicated by a dashed line. SEs are shown in all instances (n = 12).

II (SD) and III (*26*). In sea urchin eggs, R deteriorated cell cycle checkpoints, and G with its adjuvants inhibited hatching enzyme transcription synergistically (*27, 28*). Recently, it was shown in this model to activate the DNA damage checkpoint CDK1/cycline B of the first cell cycle of development (*29, 30*) for commitment to cell death by apoptosis in the case of failure of DNA repair.

This work focuses on the cell death mechanism in human cells induced by four different G formulations with a large number of agricultural applications. We have chosen Roundup Express (R7.2), Roundup Bioforce or Extra 360 (R360), Roundup Grand Travaux (R400), and Roundup Grand Travaux Plus (R450) at subagricultural dilutions. We tested them on three important enzymatic biomarkers. First, at the membrane level, we measured adenylate kinase (AK) activity after its release in the medium (*31*), revealing cytoplasmic membrane rupture, corresponding to a necrosis and/or a secondary necrosis at the end of apoptosis (*32*). Second, at the mitochondrial respiration level, we measured succinate dehydrogenase (SD) activity (*33*). Third, we tested the cytosolic level with caspase 3 and 7 activities to determine the apoptosis pathway (*34*−*36*) and in situ DNA fragmentation (DAPI). Necrosis is evinced by cytoplasmic swelling, rupture of the plasma membrane, swelling of cytoplasmic organelles (particularly mitochondria), and some condensation of nuclear chromatin, whereas apoptosis is manifested by cytoplasmic and nuclear condensation (pyknosis), nuclear fragmentation (karyorrhexis), normal morphological appearance of cytoplasmic organelles, and an intact plasma membrane; following nuclear fragmentation, the cell disaggregates into a number of membrane-bound apoptotic bodies (*37, 32*). By contrast, cell death is now known to be perpetrated through a variety of mechanisms. It can be classified into four different types, based upon morphological characteristics: apoptosis (type 1), autophagy (type 2), necrosis (oncosis, type 3), and mitotic catastrophe (*37*).

The three human cell types allowed us to establish not only the differential sensitivity of these models but also the general human cell pathways of G-based pesticides actions from 1 ppm (0.0001%); these were produced by G itself, its major metabolite AMPA, and the main adjuvant POEA, singly or in combination.

Materials and Methods

Chemicals. *N*-Phosphonomethyl glycine (glyphosate, G, PM 169.07) and its major metabolite AMPA (PM 111.04) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Herbicide Roundup formulations (Monsanto, Anvers, Belgium) were available on the market: Roundup Express 7.2 g/L of G, homologation 2010321 (R7.2); Bioforce or Extra 360 at 360 g/L of G, homologation 9800036 (R360); Grands Travaux 400 g/L of G, homologation 8800425 (R400); and Grands Travaux plus 450 g/L of G, homologation 2020448 (R450). A 2% solution of Roundup (1 or 2% is recommended by the company for agricultural use) and an equivalent solution of glyphosate to Roundup Bioforce were prepared in serum-free medium and

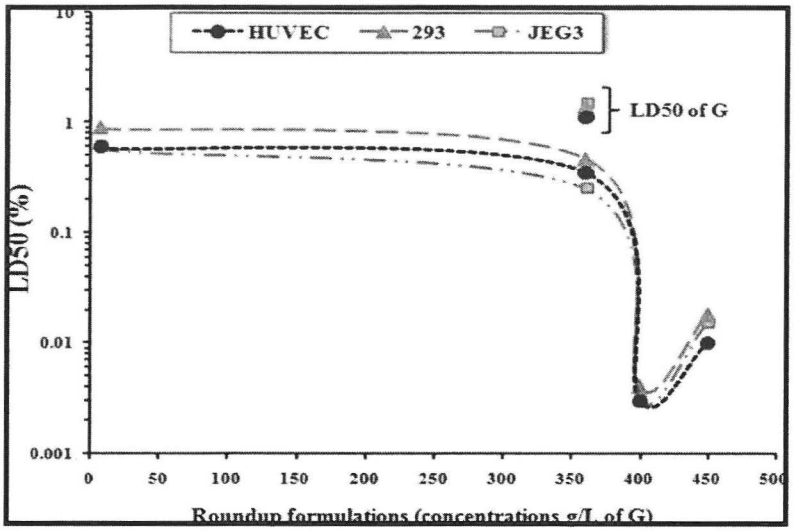

Figure 2. Nonlinear dose effects of R formulations. The LD_{50} (%) measured by SD are compared for the 4 R (see the Figure 1A legend) and G for the three cell types in similar conditions.

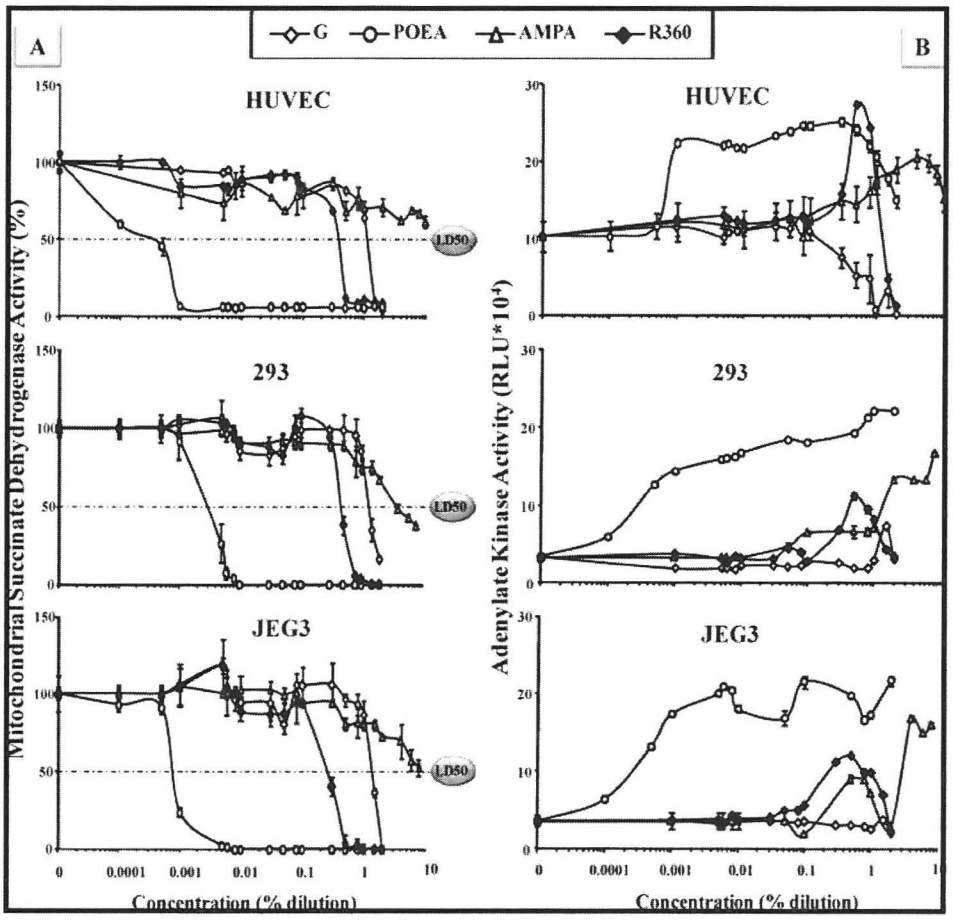

Figure 3. Cytotoxicity of R adjuvant (POEA) and glyphosate (G) metabolite (AMPA) on three human cell types. G and R360 were used as controls in similar conditions as in Figure 1 (see legend), in comparison to R adjuvant POEA and G metabolite AMPA ($1-10^5$ ppm). The 50% lethal dose (LD_{50}) is indicated by a dashed line. SEs are shown in all instances ($n = 12$).

adjusted to pH 5.8 of the 2% Roundup Bioforce solution. The major adjuvant of Roundup, polyethoxylated tallowamine (POEA at 785 g/L), was a gift from Pr. Robert Bellé (UMR 7150 CNRS/UPMC, Station Biologique de Roscoff, France). Successive dilutions were then obtained with serum-free medium. 4′,6′-Diamidino-2-phenylindole, dihydrochloride (DAPI) nucleic acid stain powder was obtained from Lonza (Saint Beauzire, France). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other compounds, otherwise precised, were obtained from Sigma-Aldrich. MTT was prepared as a 5 mg/mL stock solution in phosphate-buffered saline, filtered through a 0.22 μm filter before use, and diluted to 1 mg/mL in a serum-free medium.

Cell Cultures. Human Primary Cells. The human primary cells used in this work were HUVEC (C2519A) provided by Lonza. Cells (passage 5 or 6) were grown according to the supplier, in specific endothelial growth medium EGM-2 SingleQuots (CC-4176) containing hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphoteri-

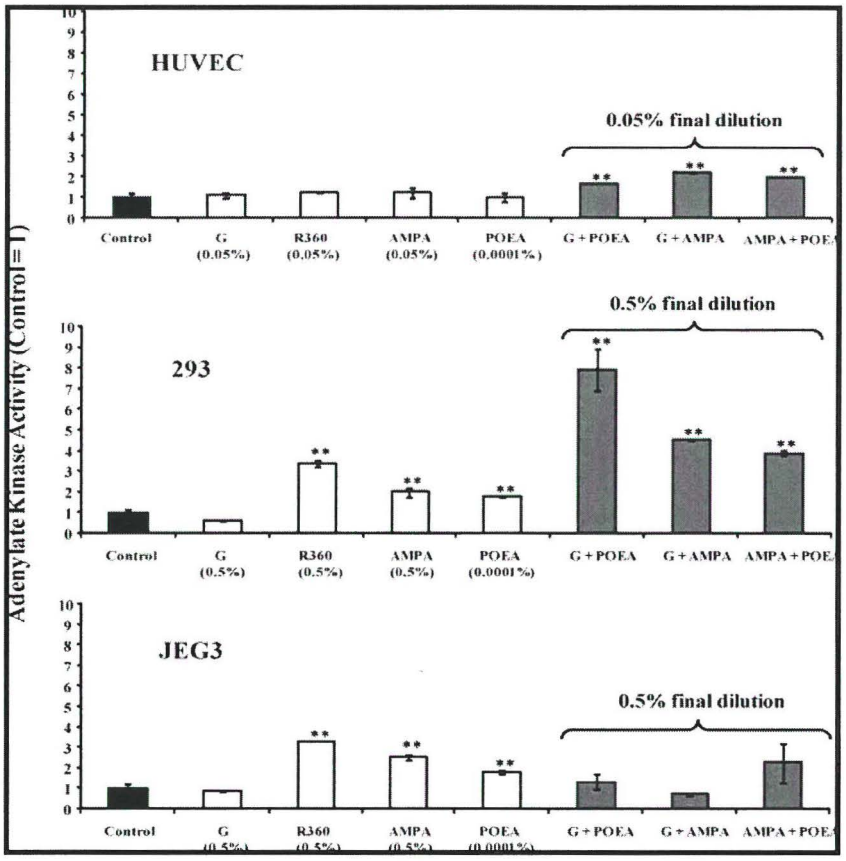

Figure 4. Combined effects of G, AMPA, and POEA on three human cell types. The cells were incubated in serum-free medium for 24 h, and the products were tested by pairs to a final concentration, where they are nontoxic alone on succinate dehydrogenase, of 0.05 (HUVEC) and 0.5% (293, JEG3). Results of cellular death are evaluated through AK activity in relative units in comparison to nontreated cells (control = 1), and values are blank-subtracted (blank = no AK); see the Materials and Methods. R360 and G are used as controls. SEs are shown in all instances (n = 16; $^{**}p < 0.01$).

cin-B), FBS (fetal bovine serum), VEGF, hFGF-B, R3-IGF-1, ascorbic acid, and heparin. Fifty thousand cells per well were grown at 37 °C (5% CO_2, 95% air) over a 24 h period to 80% confluence in 48 well plates and were washed with serum-free EGM-2.

Human Cell Lines. The human embryonic kidney 293 cell line (ECACC 85120602) and the human choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308) were provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France) containing 2 mM glutamine, 1% nonessential amino acid, 100 U/mL antibiotics (a mix of penicillin, streptomycin, and fungizone; Lonza), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France), and 10% FBS (PAA, les Mureaux, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate. Fifty thousand cells per well were grown at 37 °C (5% CO_2, 95% air) over a 48 h period to 80% confluence in 48 well plates and were washed with serum-free EMEM.

Cell Treatments. Cells were exposed for 24 h in serum-free medium to various dilutions of the different treatments including the four Roundup formulations (R7.2, R360, R400, and R450), G, AMPA, or POEA (14 concentrations from 10 ppm to 2%) and, particularly for POEA, were tested at the very low concentrations of 1 and 5 ppm; for AMPA, we tested in addition 4, 6, 8, and 10%. In another case, cells were incubated with G, AMPA, and POEA mixtures by pairs at the final nontoxic dilution on SD of 0.5% on the human cell lines (293 or JEG3) and 0.05% on the human primary cells (HUVEC) in comparison to R360.

For the details, in each cell type, three combinations were studied. For the two cell lines, the first mixture was the combination of G (0.4999%) with POEA (0.0001%); the second was the combination of G (0.4%) with AMPA (0.1%), and the third was AMPA (0.4999%) plus POEA (0.0001%). For the primary HUVEC cells, the first mixture was G (0.04999%) with POEA (0.0001%); the second was G (0.04%) with AMPA (0.01%), and the third was AMPA (0.04999%) plus POEA (0.0001%).

Cell Death Measurements. Mitochondrial Activity Measurement. This measure was based on the cleavage of MTT into a blue-colored product (formazan) by the mitochondrial enzyme succinate dehydrogenase (*38, 39, 33*); it was used to evaluate human cell viability. After cell treatments, the supernatants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum-free medium and incubated with 200 μL MTT per well after each treatment. The 48 well plates were incubated for 3 h at 37 °C, and 200 μL of 0.04 N hydrochloric acid-containing isopropanol solution was added to each well. The plates were then vigorously shaken to solubilize the blue formazan crystals formed. The optical density was measured at 570 nm using a luminometer Mithras LB 940 (Berthold, Thoiry, France).

Cell Membrane Damage Assay. The bioluminescent ToxiLight bioassay (Lonza) was a nondestructive cytotoxicity highly sensitive assay designed to measure toxicity in mammalian cells and cell lines in culture. It quantitatively measured the release of cytosolic AK from the membranes of damaged cells (*40, 31*). AK is a robust protein present in all eukaryotic cells, which is released into the culture medium when cells die, described as an important necrosis marker. The enzyme actively phosphorylated ADP, and the resultant ATP was then measured using the bioluminescent firefly luciferase reaction with the ToxiLight reagent. After 24 h of different treatments, 50 μL of cell supernatants was deposited in 96 well black plates. Then, 50 μL of the AK detection reagent (AKDR) was added by well. Plates were then placed under agitation for 15 min safe from the light, and then, luminescence was measured using the luminometer Mithras LB 940 (Berthold) at 565 nm. The serum-free medium was the negative control, and a positive control was the active reagent AKDR mixed with cells treated in the serum-free medium to determine the basal activity.

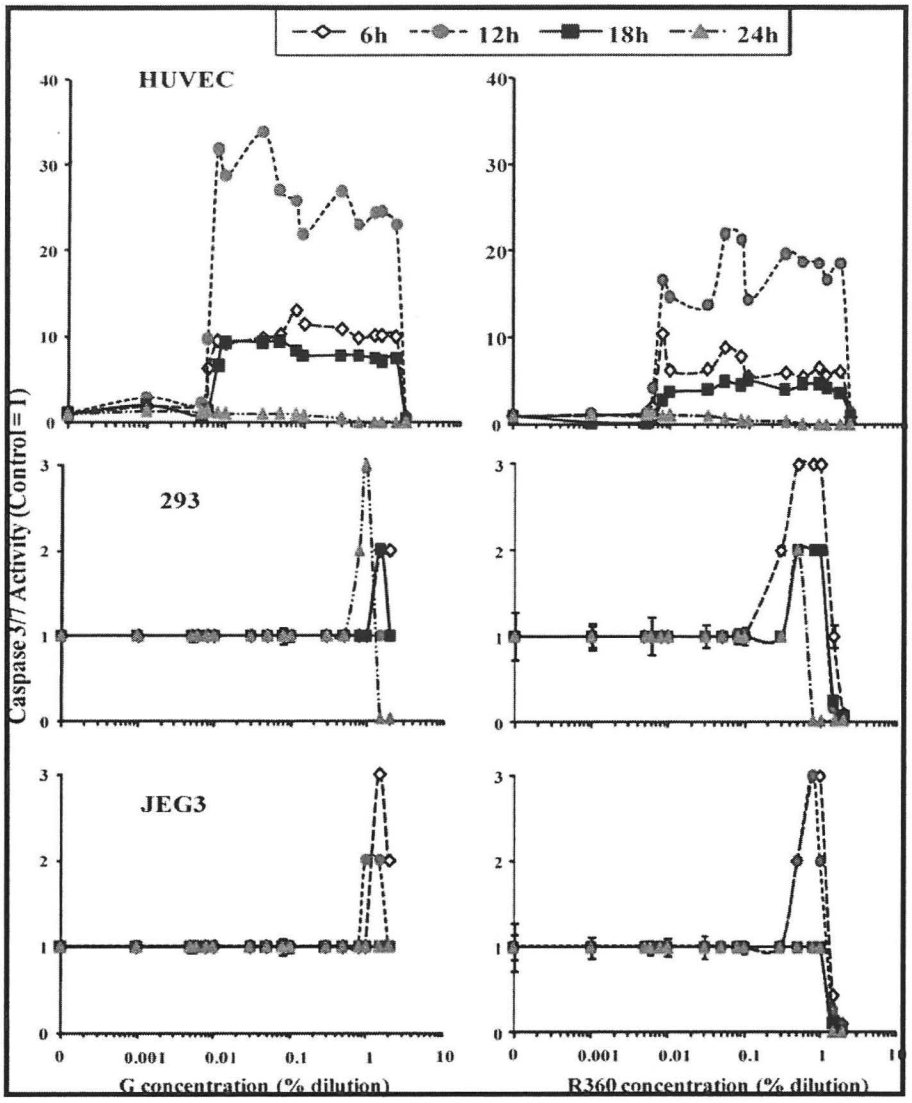

Figure 5. Time-dependent apoptosis through caspases 3/7 induction by R and G in three human cell types. R360 and G, at similar concentrations and pH (as in Figure 1), were incubated for 6, 12, 18, or 24 h. The apoptotic pathway was tested by the Caspase-Glo 3/7 assay, and results are presented in relative units to nontreated cells (control = 1). SEs are shown in all instances (n = 8).

Apoptotic Cell Death Measurements. The Caspase-Glo 3/7 assay (Promega, Paris, France) was a luminescent kit designed for automated high-throughput screening of caspases activity or apoptosis. It can measure caspase 3 and 7 activities in purified enzyme preparations or cultures of adherent or suspension cells (*41, 42, 36*). The assay provided a pro-luminescent caspase 3/7 substrate, which contains the tetrapeptide sequence DEVD active group. This substrate was cleaved to release amino-luciferin, a substrate of luciferase used in the production of light. The Caspase-Glo 3/7 reagent was optimized for caspase activity, luciferase activity, and cell lysis. The addition of the single Caspase-Glo 3/7 reagent, in an "add-mix-measure" format, resulted in cell lysis followed by caspase cleavage of the substrate and generation of a "glow type" luminescent signal. The Caspase-Glo 3/7 bioassay was carried out in 96 well white plates.

After cell cultures and their treatments by 50 μL of various dilutions, an equal volume of the reagent was added to each well. Plates were then agitated for 15 min safe from the light, to stabilize the light signal before measuring luminescence. Again, the negative control was the serum-free medium, and the positive control was the active reagent mixed with cells treated in the serum-free medium to determine the basal activity of the caspases 3/7. Luminescence was measured using the luminometer Mithras LB 940 (Berthold) at 565 nm.

Cell Microscopy. At the end of the 24 h cell treatment, the serum-free medium was removed, and cells were fixed in absolute ethanol−chloroform−acetic acid (6:3:1, v/v/v) for 1 day at −20 °C. Each well was washed with PBS (pH 7.4) and incubated with 1 μg/mL DAPI solution (*43*). Staining of DNA with DAPI was examined with a microscope using a fluorescent mode (model Leïca LMD 6000, Rueil Malmaison, France). Labeled DNA of viable cells was scattered throughout the nucleus, and bright condensation of chromatin revealed apoptotic cells (magnification, 400×). At the end of the cell treatment, the microphotographs (magnification, 100×; blue filter) of cells without coloration were also obtained with the Leïca Microscopy Systems (model Leïca DC 100, Germany).

Statistical Analysis. The experiments were repeated at least three times during different weeks on three independent cultures each time. All data were presented as the means ± standard errors (SEs). Statistical differences were determined by a Student's t test using significant levels of 0.01 (**).

Results

We have studied for the first time the mechanism of cellular action of different R on human cells, from placenta, embryonic kidney, and neonate. The first surprising results show that the four R herbicides and G cause cellular death for all types of human cells, with comparable toxicity for each one but at different concentrations. For instance, 20 ppm for R400 at 24 h,

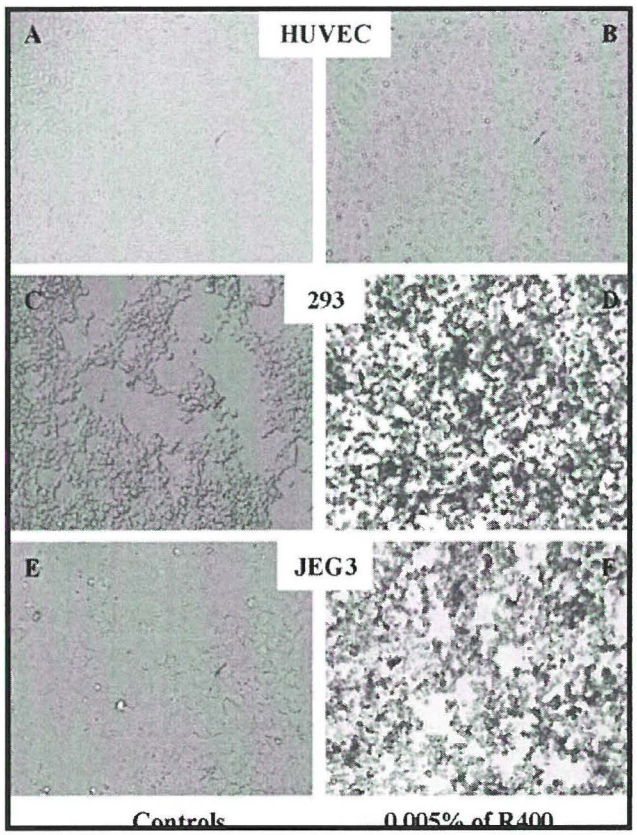

Figure 6. Microphotographs of R-treated human cells. The cell types were without coloration (magnification, 100×; blue filter), HUVEC (A, B), 293 (C, D), and JEG3 (E, F), and were incubated with 0.005% of R400 or not (controls) in serum-free medium for 24 h. Microphotographs were obtained with the Leïca Microscopy Systems (model Leïca DC 100).

the most toxic, corresponds approximately to 47 μM G (8 ppm) with adjuvants (Figure 1). However, 4−10 ppm G alone is nontoxic; its toxicity begins around 1%. The mechanism is constant for all R: There is a release of AK, indicative of cell membrane damage, and an inhibition of the mitochondrial SD (Figure 1). For all R, the membrane damage (AK) is 1.5−2 times more sensitive than mitochondrial activity (SD) for 293 and JEG3 or equally sensitive for HUVEC. By contrast, G induces mitochondrial toxicity without cell membrane damage. Unexpectedly, R400 is more toxic than another formulation containing more G, such as R450; the latter is in turn more harmful than R360, R7.2, and G in last, but all of them are detrimental nonproportionally to the G concentration that they contain. This is illustrated in Figure 2.

The mitochondrial SD inhibition measures cell asphyxia. It is obvious from Figure 2 that 7.2 or 360 g/L G with adjuvants in R formulations has closely comparable actions on cell death, while 400 or 450 g/L gives inversely proportional effects in another range. This is not an artifact since the embryonic and placental cell lines behave remarkably similarly in that regard and the primary umbilical cord cells have sensitivity for all R and G just analogous to these cell lines (Figure 2). The mortality in all cases is not linearly linked to G. The hypothesis that other substances are implicated has thus to be investigated in the formulation of the product.

Consequently, the major G metabolite, AMPA, and the surfactant POEA, the main claimed adjuvant by the manufacturer (the exact composition is a secret of formulation), have been tested separately in a first approach, in comparison to G and R360 as controls, and in similar conditions as in Figure 1, from very low subagricultural dilutions (10^{-6} if used pure like claimed by some farmers and 10^{-4} if diluted as recommended at 1%).

G is claimed by the manufacturer to be the active ingredient, and it is claimed to be not toxic for human cells but toxic for vegetable ones when mixed with inert components. Our study demonstrates for the first time that all products including AMPA and POEA provoke SD and AK effects in human cells, and thus mortality (Figure 3), but at different concentrations. Astonishingly, the supposed inert product POEA is the most potent one. From 1 ppm, it begins to alter SD in HUVEC and AK in 293 and JEG3. The mixture R is then more poisonous than G or AMPA. The metabolite AMPA itself destroys the cell membrane (AK release), whatever the cell type. This is not observed with G, which is, however, 3−8 times more inhibitory on SD than AMPA, with some differences between cells. However, because the cell membrane damage is generally more sensitive, the metabolite AMPA is finally more toxic than G on human cells. POEA is the most toxic; if it was the only adjuvant of R360, its maximal concentration would be around 1−24 ‰, according to the cells. Thus, POEA could be considered as the active ingredient on human cell death and more damaging than G. As R is more viscous than 1‰ POEA plus G, it is obvious that other compounds are in the mixture.

Thus, it was necessary to study the combined effects on cell membrane integrity (by AK release). We have tested the compounds by pairs at maximal levels where alone they do not influence SD (Figure 4). This was to assess the respective role of each one, knowing that R contains all tested compounds when metabolized. In contrast to previous results, the cells reacted differently. The mixtures were more disrupting on embryonic and umbilical cells, respectively, while placental carcinoma cells appeared to be more membrane-resistant but to mixtures only. It is very clear that if G, POEA, or AMPA has a small toxic effect on embryonic cells alone at low levels, the combination of two of them at the same final concentration is significantly deleterious (Figure 4).

We have thus elucidated that R- and G-induced cell death can be due, at least in part, to apoptosis via caspases 3/7 induction (Figure 5). The caspases are activated from 6 h with a maximum at 12 h in all cases, but umbilical primary cells are 60−160 times more sensitive than lines (293 and JEG3, respectively) at this level. Moreover, G and R360 enhance exactly at the same concentration caspases, from 50 ppm (HUVEC). The adjuvants do not appear to be necessary to render G as a death inducer at this level. Even G alone is 30% more potent on this pathway than R. Surprisingly, G acted very rapidly at concentrations 500−1000 times lower than agricultural use on human cell apoptosis. This apoptotic pathway was also activated at levels 200 times lower for G on caspases than its action on SD for umbilical cells, and for R at levels 60 times lower, in a four times shorter period (6−24 h). After 24 h of treatment, the caspases returned to basal level when SD and AK react significantly. These data are consistent with a gradual loss of caspases 3/7 activity in apoptotic cells that undergo secondary necrosis in vitro (*44*).

Our results are confirmed by the morphology of the cells after treatment by R (for instance R400, Figure 6B,D,E) in comparison with the normal cell types (A, C, F). Indeed, the very weak R concentration of 0.005% causes a very important cell death, lack of adhesion, shrinking, and fragmentation in apoptotic bodies. This is confirmed in Figure 7 with the DNA fluorescent labeling with DAPI, for example, with R360 at 0.5% over 24 h. The characteristic fluorescence of apoptotic cells evidencing

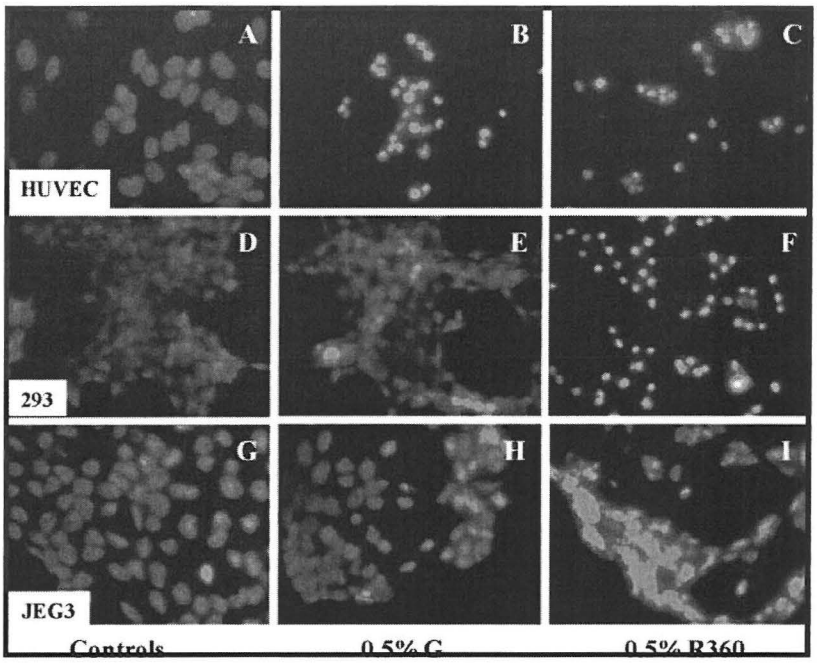

Figure 7. Increase of DNA condensation (DAPI test) in R360- or G-treated human cells. The cell types HUVEC (A−C), 293 (D−F), and JEG3 (G−I) were incubated for 24 h with or without 0.5% R360 or G at equivalent concentrations. Staining of DNA with DAPI was examined with a microscope model Leïca LMD 6000, using a fluorescent mode. Labeled DNA of viable cells was scattered throughout the nucleus, and bright condensation of chromatin revealed apoptotic cells (magnification, 400×).

DNA condensation is more visible with the herbicide than in controls (A, D, G) and more after R treatment (C, F, I) than with G alone (B, E, H), for cell lines. The primary cells are similarly sensitive to G than to R, as for caspases activation in Figure 5.

Discussion

We had previously demonstrated (*14*) that G-based formulations were able to affect human placental cell viability at subagricultural doses (0.1% in 18 h) and sexual steroid biosynthesis at lower nontoxic doses (0.01%) and that this was due at least in part to G, but its action was highly amplified by adjuvants, the so-called inert ingredients of R formulations, kept confidential by the companies (*9*). However, the question of a specific cell line action or a time reversible effect remained open. Benachour et al. (*15*) demonstrated that in embryonic cells as well as in normal human placenta and equine testis, there was a similar G-dependent endocrine disruption, through aromatase inhibition, at nontoxic levels. The embryonic cells were even more sensitive: It was discovered that the cell mitochondrial activity was also reached in time- and dose-dependent manners by the G formulation R360. The cytotoxicity was amplified around 14 times between 24 and 72 h (*15*), suggesting either a bioaccumulation or a time-delayed effect and suggesting a cumulative impact, after endocrine disruption, of very low doses around G acceptable daily intake (ADI: 0.3 mg/kg/j), according to the nature of the adjuvants.

To understand in vivo effects through the interpretation of the in cell impacts described above, it is necessary to have knowledge of the dilution and of the processes leading to an elimination of the product in the body. This must be taken into account in regard to its bioaccumultation potential and time-delayed effects. This is why we have measured the caspases activities at different times and G or R concentrations, after having previously demonstrated their effects amplified with time within 3 days, on SD in embryonic and placental cells (*15*). Moreover, the metabolism of the herbicide has to be considered, and the tests in this study of all the above-cited products approach this question.

All cell types, including primary cultures, react similarly at the membrane and mitochondrial level, justifying the hypothesis that the cell lines used provide excellent models to study human cell toxicity, for instance in placental cells (*18*). We show for the first time that embryonic and umbilical cells also have comparable sensitivity. The most reactive level reached appears to be the cell membrane level for the different formulations, but not for G. The supposed "inert ingredients" play obviously and differently the role of cell membrane disruptors, independently to G, as we have previously proposed (*14*), and this was suggested in fish, amphibians, and microorganisms (*27, 45*) or in plants (*46*). We now demonstrate that in human cells.

The second level is the mitochondrial membrane and the enzymatic reaction in it, SD, localized in the internal membrane in complex II of the respiratory chain (*47*). It is altered in a comparable way, not proportional to G but relatively to the nature and the quantity of the adjuvants that we have previously listed (*15*). This means that the toxicity of G clearly varies with formulations that must imperatively now be used in in vivo tests to study any toxicity (*45*); this also means that the ADI of G must take into account its formulation, since 7.2 or 360 g/L of G may have comparable effects, considerably different to 400 g/L. It would even be more correct to use precisely an ADI of R instead of G. It may also be time-dependent. These ideas are not taken into account yet for regulatory legislation.

The necessity to study combined effects also appears from our results. In fact, the body is always exposed to mixtures and not to single compounds. We have previously demonstrated that mixtures could amplify toxicity for other widely spread pollutants (*2*). For embryonic or neonatal cells, POEA, the major adjuvant, has the highest toxicity, either by itself or amplified 2−5 times in combination with G or AMPA. It has already been shown that POEA is highly toxic for sea urchin embryos, impinging on transcription (*28*). It is also known that in an

aquatic environment, POEA has higher effects than R and G on bacteria, microalgae, protozoa, and crustaceans (*12*). In addition, the known metabolism of G in the soil or plants is supposed to detoxify it in AMPA (*11*); however, here, we demonstrate that AMPA is more toxic than G in human cells, especially on cell membrane. AMPA is also more stable in soil (*48*), in plants, and in food or feed residues (*49*), and more present in wastewater (2−35 ppm) than G [0.1−3 ppm; (*50*)]. It is not toxic alone at these concentrations in our experiments, but it amplifies G or POEA toxicity in combination. The synergic toxicity of all of these compounds is now more obvious.

The induced resistance of placental cancer cells (*51*) could explain a specific difference for JEG3 cells at these levels. The placental cells could form an efficient barrier to mixtures before their death, since the membranes are more resistant, and this could be due to the fact that there are carcinoma-derived cells that have acquired a capacity to excrete xenobiotics.

The caspases 3/7 inducing apoptosis were in fact activated first within 6 h, and then, they decreased with cell mortality. This corroborates the timing observed for another compound (*36*). The caspases induction by G alone is observed at doses that do not provoke cell or mitochondrial membrane damages, indicating a clear G-apoptotic pathway always at subagricultural doses. Mixed with adjuvants, G in R formulations reached the other end points. This suggests that the adjuvants could also play a role in total cell death, through necrosis characterized by organelle alterations with mitochondrial and cell membranes swelling and ruptures (*52*). The most sensitive are umbilical HUVEC cells, for which apoptosis has been described (*53*−*55*), but very rarely induced by a pesticide, for example, in the case of diallyl trisulfide (*56*). Surprisingly, this phenomenon was observed for G and R at similar and low concentrations, as if a cell membrane death receptor was activated (*57, 32*), with no G penetration necessary. The modification of a dependency receptor is another pathway that could be studied (*58*). The apoptotic cell appearance was microscopically confirmed. Then, our next step could be to study the necrotic/apoptotic ratio within short times.

In conclusion, mixtures called "formulations" change cell permeability, toxicity, and pathways of xenobiotics: In all cases, cell death is induced more by R than by AMPA or G, and the latter provokes apoptosis (from 50 ppm in HUVEC cells) without membrane damage. By contrast, G mixed with adjuvants in R formulations disrupts cell and mitochondrial membranes and promotes necrosis. It becomes obvious that the "threshold" level of action of the herbicide should take into account the period and length of exposure, the presence of adjuvants, in particular POEA, metabolism, and bioaccumulation or time-delayed effects. All of the above effects are demonstrated below the recommended herbicide agricultural dilutions (from 10^4 ppm). This clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

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References

(1) Feron, V. J., Cassee, F. R., Groten, J. P., van Vliet, P. W., and van Zorge, J. A. (2002) International issues on human health effects of exposure to chemical mixtures. *Environ. Health Perspect. 110*, 893–899.
(2) Benachour, N., Moslemi, S., Sipahutar, H., and Séralini, G. E. (2007) Cytotoxic effects and aromatase inhibition by xenobiotic endocrine distrupters alone and in combination. *Toxicol. Appl. Pharmacol. 222*, 129–140.
(3) Tichy, M., Borek-Dohalsky, V., Rucki, M., Reitmajer, J., and Feltl, L. (2002) Risk assessment of mixtures: Possibility of prediction of interaction between chemicals. *Int. Arch. Occup. Environ. Health 75*, S133−S136.
(4) Monosson, E. (2005) Chemical mixtures: Considering the evolution of toxicology and chemical assessment. *Environ. Health Perspect. 113*, 383–390.
(5) Acquavella, J. F., Bruce, H., Alexander, B. H., Mandel, J. S., Gustin, C., Baker, B., Champan, P., and Bleeke, M. (2004) Glyphosate biomonitoring for farmers and their families: Results from the farm family exposure study. *Environ. Health Perspect. 112*, 321–326.
(6) Takahashi, M., Horie, M., and Aoba, N. (2001) Analysis of glyphosate and its metabolite, aminomethylphosphonic acid, in agricultural products by HPLC. *Shokuhin Eiseigaku Zasshi 42*, 304–308.
(7) Clive, J. (2007) The global status of the commercialized biotechnological/genetically modified crops: 2006. *Tsitol. Genet. 41*, 10–12.
(8) IFEN (2006) Report on pesticides in waters: Data 2003−2004. Institut Français de l'Environnement, Orleans, France. *Dossier 5*, 15–20.
(9) Cox, C. (2004) Glyphosate. *J. Pest. Reform. 24*, 10–15.
(10) Acquavella, J. F., Weber, J. A., Cullen, M. R., Cruz, O. A., Martens, M. A., Holden, L. R., Riordan, S., Thompson, M., and Farmer, D. (1999) Human ocular effects from self-reported exposures to Roundup herbicides. *Hum. Exp. Toxicol. 18*, 479–86.
(11) Williams, G. M., Kroes, R., and Munro, I. C. (2000) Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for human. *Regul. Toxicol. Pharmacol. 31*, 117–65.
(12) Tsui, M. T., and Chu, L. M. (2003) Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere 52*, 1189–1197.
(13) Cox, C. (1998) Glyphosate (Roundup). *J. Pest. Reform. 18*, 3–17.
(14) Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., and Séralini, G. E. (2005) Differential effects of glyphosate and Roundup on human placental cells and aromatase. *Environ. Health Perspect. 113*, 716–720.
(15) Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., and Séralini, G. E. (2007) Time and dose-dependent effects of Roundup on human embryonic and placental cells and aromatase inhibition. *Arch. Environ. Contam. Toxicol. 53*, 126–133.
(16) Savitz, D., Arbuckle, T., Kaczor, D. M., and Curtis, K. (1997) Male pesticide exposure and pregnancy outcome. *Am. J. Epidemiol. 146*, 1025–1036.
(17) Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., Van der Saag, P. T., Van der Burg, B., and Gustafsson, J. A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology 139*, 4252–4263.
(18) Letcher, R. J., Van Holstein, I., Drenth, H. J., Norstrom, R. J., Bergman, A., Safe, S., Pieters, R., and van den Berg, M. (1999) Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. *Toxicol. Appl. Pharmacol. 160*, 10–20.
(19) L'Azou, B., Fernandez, P., Bareille, R., Beneteau, M., Bourget, C., Cambar, J., and Bordenave, L. (2005) In vitro endothelial cell susceptibility to xenobiotics: Comparison of three cell types. *Cell Biol. Toxicol. 21*, 127–137.
(20) Thorin, E., and Shreeve, S. M. (1998) Heterogeneity of vascular endothelial cells in normal and disease states. *Pharmacol. Ther. 78*, 155–166.
(21) Peters, R. J. B. (2005) Man-Made Chemicals in Maternal and Cord Blood. TNO report B&O-A R 2005/129.
(22) Annas, A., Granberg, A. L., and Brittebo, E. B. (2000) Differential response of cultured human umbilical vein and artery endothelial cells to Ah receptor agonist treatment: CYP-dependent activation of food and environmental mutagens. *Toxicol. Appl. Pharmacol. 169*, 94–101.
(23) Choi, W., Eum, S. Y., Lee, Y. W., Hennig, B., Robertson, L. W., and Toborek, M. (2003) PCB 104-induced proinflammatory reactions in human vascular endothelial cells: Relationship to cancer metastasis and atherogenesis. *Toxicol. Sci. 75*, 47–56.
(24) Rathinasamy, K., and Panda, D. (2006) Suppression of microtubule dynamics by benomyl decreases tension across kinetochore pairs and induces apoptosis in cancer cells. *FEBS J. 273*, 4114–4128.
(25) Kannan, K., Holcombe, R. F., Jain, S. K., Alvarez-Hernandez, X., Chervenak, R., Wolf, R. E., and Glass, J. (2000) Evidence for the

induction of apoptosis by endosulfan in a human T-cell leukemic line. *Mol. Cell. Biochem. 205*, 53–66.
(26) Peixoto, F. (2005) Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere 61*, 1115–1122.
(27) Marc, J., Mulner-Lorillon, O., Boulben, S., Hureau, D., Durand, G., and Bellé, R. (2002) Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. *Chem. Res. Toxicol. 15*, 326–331.
(28) Marc, J., Le Breton, M., Cormier, P., Morales, J., Bellé, R., and Mulner-Lorillon, O. (2005) A glyphosate-based pesticide impinges on transcription. *Toxicol. Appl. Pharmacol. 203*, 1–8.
(29) Bellé, R., Le Bouffant, R., Morales, J., Cosson, B., Cormier, P., and Mulner-Lorillon, O. (2007) Sea urchin embryo, DNA-damaged cell cycle checkpoint and the mechanisms initiating cancer development. *J. Soc. Biol. 201*, 317–27.
(30) Le Bouffant, R., Cormier, P., Cueff, A., Bellé, R., and Mulner-Lorillon, O. (2007) Sea urchin embryo as a model for analysis of the signaling pathways linking DNA damage checkpoint, DNA repair and apoptosis. *Cell. Mol. Life Sci. 64*, 1723–1734.
(31) Squirrell, D., and Murphy, J. (1997) Rapid detection of very low numbers of micro-organisms using adenylate kinase as a cell marker. A Practical Guide to Industrial Uses of ATP Luminescence in Rapid Microbiology, pp 107−113 Cara Technology Ltd., Lingfield, Surrey, United Kingdom.
(32) Taatjes, D. J., Sobel, B. E., and Budd, R. C. (2008) Morphological and cytochemical determination of cell death by apoptosis. *Histochem. Cell. Biol. 129*, 33–43.
(33) Scatena, R., Messana, I., Martorana, G. E., Gozzo, M. L., Lippa, S., Maccaglia, A., Bottoni, P., Vincenzoni, F., Nocca, G., Castagnola, M., and Giardina, B. (2004) Mitochondrial damage and metabolic compensatory mechanisms induced by hyperoxia in the U-937 cell line. *J. Biochem. Mol. Biol. 37*, 454–459.
(34) Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1999) The role of caspases in development, immunity, and apoptotic signal transduction: Lessons from knockout mice. *Immunity 10*, 629–639.
(35) Omezzine, A., Chater, S., Mauduit, C., Florin, A., Tabone, E., Chuzel, F., Bars, R., and Benahmed, M. (2003) Long-term apoptotic cell death process with increased expression and activation of caspase-3 and -6 in adult rat germ cells exposed in utero to flutamide. *Endocrinology 144*, 648–661.
(36) Liu, J. J., Wang, W., Dicker, D. T., and El-Deiry, W. S. (2005) Bioluminescent imaging of TRAIL-induced apoptosis through detection of caspase activation following cleavage of DEVD-aminoluciferin. *Cancer Biol. Ther. 4*, 885–892.
(37) Galluzzi, L., Maiuri, M. C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., and Kroemer, G. (2007) Cell death modalities: Classifications and pathophysiological implications. *Cell Death Differ. 14*, 1237–43.
(38) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65*, 55–63.
(39) Denizot, F., and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods 89*, 271–277.
(40) Crouch, S. P. M., Kozlowski, R., Slater, K. J., and Fletcher, J. (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods 160*, 81–88.
(41) O'Brien, M., Moravec, R., Riss, T., and Promega Corporation (2003) Caspase-Glo 3/7 assay: Use fewer cells and spend less time with this homogeneous assay. *Cell Notes 6*, 13−15.
(42) Ren, Y. G., Wagner, K. W., Knee, D. A., Aza-Blanc, P., Nasoff, M., and Deveraux, Q. L. (2004) Differential regulation of the TRAIL death receptors DR4 and DR5 by the signal recognition particle. *Mol. Biol. Cell 15*, 5064–5074.
(43) Travert, C., Carreau, S., and Le Goff, D. (2006) Induction of apoptosis by 25-hydroxycholesterol in adult rat Leydig cells: Protective effect of 17beta-estradiol. *Reprod. Toxicol. 22*, 564–570.
(44) Riss, T. L., and Moravec, R. A. (2004) Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev. Technol. 2*, 51–62.
(45) Cox, C., and Surgan, M. (2006) Unidentified inert ingredients in pesticides: Implications for human and environmental health. *Environ. Health Perspect. 114*, 1803–806.
(46) Haefs, R., Schmitz-Eiberger, M., Mainx, H. G., Mittelstaedt, W., and Noga, G. (2002) Studies on a new group of biodegradable surfactants for glyphosate. *Pest. Manage. Sci. 58*, 825–833.
(47) Thomas, P. K., Cooper, J. M., King, R. H., Workman, J. M., Schapira, A. H., Goss-Sampson, M. A., and Muller, D. P. (1993) Myopathy in vitamin E deficient rats: Muscle fibre necrosis associated with disturbances of mitochondrial function. *J. Anat. 183*, 451–461.
(48) Gard, J. K., Feng, P. C., and Hutton, W. C. (1997) Nuclear magnetic resonance timecourse studies of glyphosate metabolism by microbial soil isolates. *Xenobiotica 27*, 633–644.
(49) Scalla, R. (1997) Sécurité alimentaire biotransformation des herbicides par les plantes transgéniques résistantes. Oléagineux, Corps Gras, Lipides. *Dossier: Génie Génét. Oléagineux 4*, 113–119.
(50) Ghanem, A., Bados, P., Kerhoas, L., Dubroca, J., and Einhorn, J. (2007) Glyphosate and AMPA analysis in sewage sludge by LC-ESI-MS/MS after FMOC derivatization on strong anion-exchange resin as solid support. *Anal. Chem. 79*, 3794–801.
(51) Xu, J., Peng, H., and Zhang, J. T. (2007) Human multidrug transporter ABCG2, a target for sensitizing drug resistance in cancer chemotherapy. *Curr. Med. Chem. 14*, 689–701.
(52) Okada, H., and Mak, T. W. (2004) Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat. Rev. Cancer 4*, 592–603.
(53) Solovyan, V. T., and Keski-Oja, J. (2005) Apoptosis of human endothelial cells is accompanied by proteolytic processing of latent TGF-beta binding proteins and activation of TGF-beta. *Cell Death Differ. 12*, 815–826.
(54) Song, Y., Shen, K., and He, C. X. (2007) Construction of autocatalytic caspase-3 driven by amplified human telomerase reverse transcriptase promoter and its enhanced efficacy of inducing apoptosis in human ovarian carcinoma. *Zhonghua Fu Chan Ke Za Zhi 42*, 617–622.
(55) Qiu, J., Gao, H. Q., Li, B. Y., and Shen, L. (2008) Proteomics investigation of protein expression changes in ouabain induced apoptosis in human umbilical vein endothelial cells. *J. Cell. Biochem. 104*, 1054–1064.
(56) Xiao, D., Li, M., Herman-Antosiewicz, A., Antosiewicz, J., Xiao, H., Lew, K. L., Zeng, Y., Marynowski, S. W., and Singh, S. V. (2006) Diallyl trisulfide inhibits angiogenic features of human umbilical vein endothelial cells by causing Akt inactivation and down-regulation of VEGF and VEGF-R2. *Nutr. Cancer 55*, 94–107.
(57) Ryu, H. Y., Emberley, J. K., Schlezinger, J. J., Allan, L. L., Na, S., and Sherr, D. H. (2005) Environmental chemical-induced bone marrow B cell apoptosis: Death receptor-independent activation of a caspase-3 to caspase-8 pathway. *Mol. Pharmacol. 68*, 1087–1096.
(58) Kroemer, G., Galluzzi, L., and Brenner, C. (2007) Mitochondrial membrane permeabilization in cell death. *Physiol. Rev. 87*, 99–163.

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Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity

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ABSTRACT

Pesticides are always used in formulations as mixtures of an active principle with adjuvants. Glyphosate, the active ingredient of the major pesticide in the world, is an herbicide supposed to be specific on plant metabolism. Its adjuvants are generally considered as inert diluents. Since side effects for all these compounds have been claimed, we studied potential active principles for toxicity on human cells for 9 glyphosate-based formulations. For this we detailed their compositions and toxicities, and as controls we used a major adjuvant (the polyethoxylated tallowamine POE-15), glyphosate alone, and a total formulation without glyphosate. This was performed after 24 h exposures on hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines. We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. The compositions in adjuvants were analyzed by mass spectrometry. Here we demonstrate that all formulations are more toxic than glyphosate, and we separated experimentally three groups of formulations differentially toxic according to their concentrations in ethoxylated adjuvants. Among them, POE-15 clearly appears to be the most toxic principle against human cells, even if others are not excluded. It begins to be active with negative dose-dependent effects on cellular respiration and membrane integrity between 1 and 3 ppm, at environmental/occupational doses. We demonstrate in addition that POE-15 induces necrosis when its first micellization process occurs, by contrast to glyphosate which is known to promote endocrine disrupting effects after entering cells. Altogether, these results challenge the establishment of guidance values such as the acceptable daily intake of glyphosate, when these are mostly based on a long term in vivo test of glyphosate alone. Since pesticides are always used with adjuvants that could change their toxicity, the necessity to assess their whole formulations as mixtures becomes obvious. This challenges the concept of active principle of pesticides for non-target species.

1. Introduction

Pesticide formulations are mixtures of adjuvants and so-called "active principles" on plants for herbicides, and insects for insecticides, etc. The supposed specificity of active principles on their targets does not mean a priori that they are the most toxic compounds of the formulations on human cells. Numerous mammalian (Colborn et al., 1993) and other animal studies (Hawthorne and Dively, 2011) evidenced side effects for pesticides. The toxicology of mixtures cannot be fully understood without knowing the differential toxicity of the various compounds of the formulations and their combined effects. Surprisingly, to measure their side effects, the active principles of pesticides are generally tested alone at a regulatory level in long-term mammalian trials, although their adjuvants are developed at least to enhance their stability and penetration into cells. However, most of the adjuvants are classified as inert.

Here we tested the differential and combined cytotoxicity of the major pesticides in the world which are glyphosate-based herbicides (GBH), and analyzed their composition and mechanisms of action. The residues of the GBH such as Roundup (R) are also among the first contaminants of ground and surface waters (IFEN, 2006), and of some food and feed because they are present since more than 15 years in around two third of genetically modified (GM) cultivated edible plants, because they are designed at least to tolerate R (James, 2011). Glyphosate (G) is toxic in plant cells by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase used as a first step in aromatic amino acid synthesis (Boocock and Coggins, 1983). Adjuvants considered as inert include, according to the formulations, surfactants like POEAs (polyethoxylated alkylamines,

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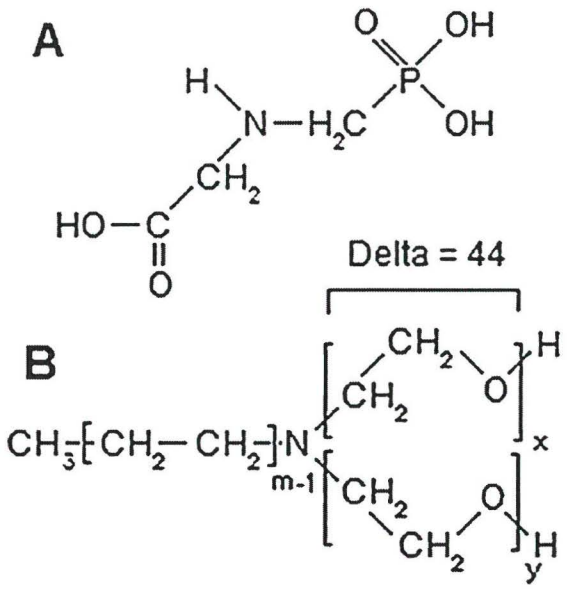

Fig. 1. Structures of glyphosate (A) and POEAs (B). Glyphosate is the N-(phosphonomethyl)glycine, C3H8NO5P). Di-ethoxylates of tallowamines adjuvants (C_mNEO_n, $n = x + y$) such as POEA are characterized by their oxide/tallowamine ratio. The delta of 44 ($—CH_2—CH_2—O—$) corresponded to the increment of the different peaks observed in mass spectrometry. Length of the more abundant tallowamine part in the adjuvant mixture corresponded to the maximal *m/z* of the spectrum.

Fig. 1), isobutane, light petroleum distillate, etc. that may induce among other DNA damages (Cox, 2004). However G is still generally hypothesized to be the active ingredient for non-target side effects. Unexpected side effects of G-based formulations were evidenced on non-target species, among other endocrine disruptions during spermatogenesis or pregnancy (Beuret et al., 2005; Clair et al., 2012; Dallegrave et al., 2007; Daruich et al., 2001; Oliveira et al., 2007; Romano et al., 2011; Savitz et al., 1997; Yousef et al., 1995). This may be related to adjuvants in formulation. They are indeed more and more considered as responsible for GBH toxicity (Mesnage et al., 2010; Williams et al., 2012), but the mechanistic and the nature of the cytotoxic agent(s) on human cells are still unknown. This is a general question that can arise for all pesticides.

The detailed known composition indicate that major adjuvants are ethoxylated, such as POEAs which are themselves mixtures of di-ethoxylates of tallowamines characterized by their oxide/tallowamine ratio. POEA commonly used in GBH is the POE (15) tallowamine (POE-15). We thus compared the toxicity and the composition of 9 formulations varying in adjuvants contents: Roundup Ultra, Roundup GT, Roundup GT+, Roundup Bioforce, Roundup 3plus, Glyphogan, Topglypho 360, Clinic E.V., and Bayer GC. For controls, we tested a formulation containing POE-15 without G (Genamin T200), and POE-15 alone. The compositional analysis of these products was performed by a non-quantitative mass spectrometry (MALDI-TOF MS/MS), considered as the best way to analyze pesticides formulations (Corbera et al., 2010; Cserháti and Forgács, 1997). Physico-chemical properties of POE-15 were approached by the measurements of its critical micelle concentration (CMC), determined by absorption changes in its presence of Coomassie blue CBB R-250.

We used HEK293, JEG3 and HepG2 cell lines, three models where unexpected effects of GBH have already been demonstrated (Benachour and Seralini, 2009; Gasnier et al., 2009). JEG3 cells are a useful model for examining placental toxicity (Letcher et al., 1999), and HepG2 for hepatic toxicity (Urani et al., 1998). HEK293 were chosen because of the sensitivity of embryonic cells, Roundup causing pregnancy outcomes (Savitz et al., 1997). Moreover, we have demonstrated that these cell lines are even less sensitive than primary cells (Benachour and Seralini, 2009; L'Azou et al., 2005), and therefore are possibly representative of a real cellular toxicity. For cytotoxicity measurements, we assayed mitochondrial succinate dehydrogenase (SD) activity (MTT assay), G and its formulations are indeed known to target mitochondria (Astiz et al., 2009; Peixoto, 2005). Cytotoxicity was also characterized by the measurement of apoptosis and necrosis, respectively by caspases 3/7 activation (Liu et al., 2005) and adenylate kinase leakage after membrane alterations (Crouch et al., 1993).

Overall, we questioned if an active toxic principle in a target species may be always generalized as such in a non target one, and thus if the regulatory toxicological tests on active principles alone are relevant.

2. Materials and methods

2.1. Chemicals

Glyphosate (N-phosphonomethyl glycine, G, CAS: 1071-83-6) was purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). GBH formulations available on the market were by alphabetical order: Bayer GC (12.5% of G, 1–5% of POE-15, homologation 05873567), Clinic EV (42% of G, 11% of POE-15, homologation 9900039), Genamin T200 (60–80% of POE-15, homologation 8500170), Glyphogan (39–43% of G, 13–18% of POE-15, homologation 9100537), Roundup Grand Travaux (400 g/L of G, R GT, homologation 8800425), Roundup Grand Travaux plus (450 g/L of G, 90 g/L of ethoxylated etheralkylamine (EtO-EA), R GT+, homologation 2020448), Roundup Ultra (41.5% of G, 16% surfactant, homologation 9700259), Roundup Bioforce (360 g/L of G, homologation 9800036), Roundup 3plus (170 g/L of G, 8% surfactant homologation 9300241), Topglypho 360 (360 g/L of G, homologation 2000254). POE-15 (CAS: 61791-26-2) was purchased from ChemService (West Chester, PA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other compounds, otherwise noticed, were obtained from Sigma–Aldrich. MTT was prepared as a 5 mg/mL stock solution in phosphate-buffered saline, filtered through a 0.22 μm filter before use, and diluted to 1 mg/mL in a serum-free medium.

2.2. Cell lines and treatments

The human embryonic kidney 293 cell line (HEK 293, ECACC 85120602), was provided by Sigma–Aldrich (Saint-Quentin Fallavier, France). The hepatoma cell line HepG2 was provided by ECACC (85011430). JEG3 cell line (ECACC 92120308) was provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM (Abcys, Paris, France) containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (a mixture of penicillin, streptomycin and fungizone) (Lonza, Saint Beauzire, France), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France) and 10% Fetal Bovine Serum (PAA, les Mureaux, France). JEG3 cells were supplemented with 1 mM sodium pyruvate. Cells were grown with this medium at 37 °C (5% CO_2, 95% air) during 48 h to 80% confluence, and then washed and exposed 24 h with serum-free EMEM to various chemicals. This model was validated (Benachour et al., 2007) since cytotoxic effects were similar in presence of serum but delayed by 48 h. The dilutions of formulated herbicides, adjuvants and G alone were prepared in serum free medium as stock solutions at a similar pH.

2.3. Cytotoxicity biomarkers

After treatments, the following tests were applied: succinate dehydrogenase (SD) activity assay (MTT) (Mosmann, 1983). Integrity of mitochondrial dehydrogenase enzymes indirectly reflects the cellular mitochondrial respiration. The optical density was measured at 570 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The bioluminescent ToxiLight bioassay (Lonza, Saint Beauzire, France) was applied for the membrane degradation assessment, by the intracellular adenylate kinase (AK) release in the medium; this is described as a necrosis marker (Crouch et al., 1993). Finally, the apoptotic cell death was evaluated with the Caspase-Glo 3/7 assay (Promega, Paris, France). Luminescence was measured using a Mithras LB 940 luminometer (Berthold, Thoiry, France). These methods were previously described (Benachour and Seralini, 2009).

2.4. Mass spectrometry (MS)

MS experiments were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF TOF ion optics and an OptiBeam™ on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of des-Arg-Bradykinin, Angiotenin I, Glu1-Fibrinopeptide B, ACTH (18–39), ACTH (7–38) and mass precision was better than 50 ppm. A 0.8 μL volume of the GBH solution diluted 100 times in water was mixed with 1.6 μL volumes

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of solutions of α-cyano-4-hydroxycinnamic acid matrix prepared in 50% ACN with 0.1% TFA. The mixture was spotted on a stainless steel Opti-TOF™ 384 targets; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. Acquisitions were taken in manual and automatic modes. A laser intensity of 3000 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 100 to 2000 Da. MS/MS spectra were acquired in the positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 3900. For the tandem MS experiments, the acceleration voltage applied was 1 kV and air was used as the collision gas. Gas pressure medium was selected as settings.

2.5. Critical micelle concentrations (CMC) determinations

CMC determinations were performed and adapted according to (Samsonoff et al., 1986). CMC was measured by the incorporation of Coomassie brilliant blue R-250 (CBB-R250) in micelles formed by serial dilutions of detergents. The CCB-R250 reagent was prepared as previously described (Bradford, 1976). Varying concentrations of adjuvants were added in a volume of 1 mL, 100 μL of CBB-R250 was added to make a final concentration of 80 μg/mL. Solutions were shaken and distributed in 96 well-plates in triplicate. Absorption was then measured against a water blank at 600 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The validation of the technique was performed with triton X-100, with a CMC of 0.15–0.20 mM (Courtney et al., 1986).

2.6. Statistical analysis

The experiments were repeated at least 3 times in different weeks on 3 independent cultures ($n = 9$). LC_{50} values were calculated by a nonlinear regression using sigmoid (5-parameters) equation with the GraphPad software. All data were presented as the means ± standard errors (SEMs). Statistical differences were determined by Student's t-test using significant levels with $p < 0.01$ (**) and $p < 0.05$ (*).

3. Results

Here we studied for the first time the precise involvement of the adjuvants and G in GBH induced toxicity, on three human cell lines from different embryonic origins (kidney, liver, and placenta) in order to test their specificities. We first compared mitochondrial respiration (SD activity) in presence of 9 formulated mixtures of G and adjuvants, G alone, formulating agents without G (Genamin), and a major adjuvant of some formulations, POE-15 (Fig. 2). All chemicals are cytotoxic, inducing similar dose-dependent patterns on HEK293, HepG2, and JEG3 in 24 h. JEG3 were up to 2-fold more sensitive to treatments than HEK293 and HepG2 in comparison to control. We observed for all cell lines different ranges of toxicities

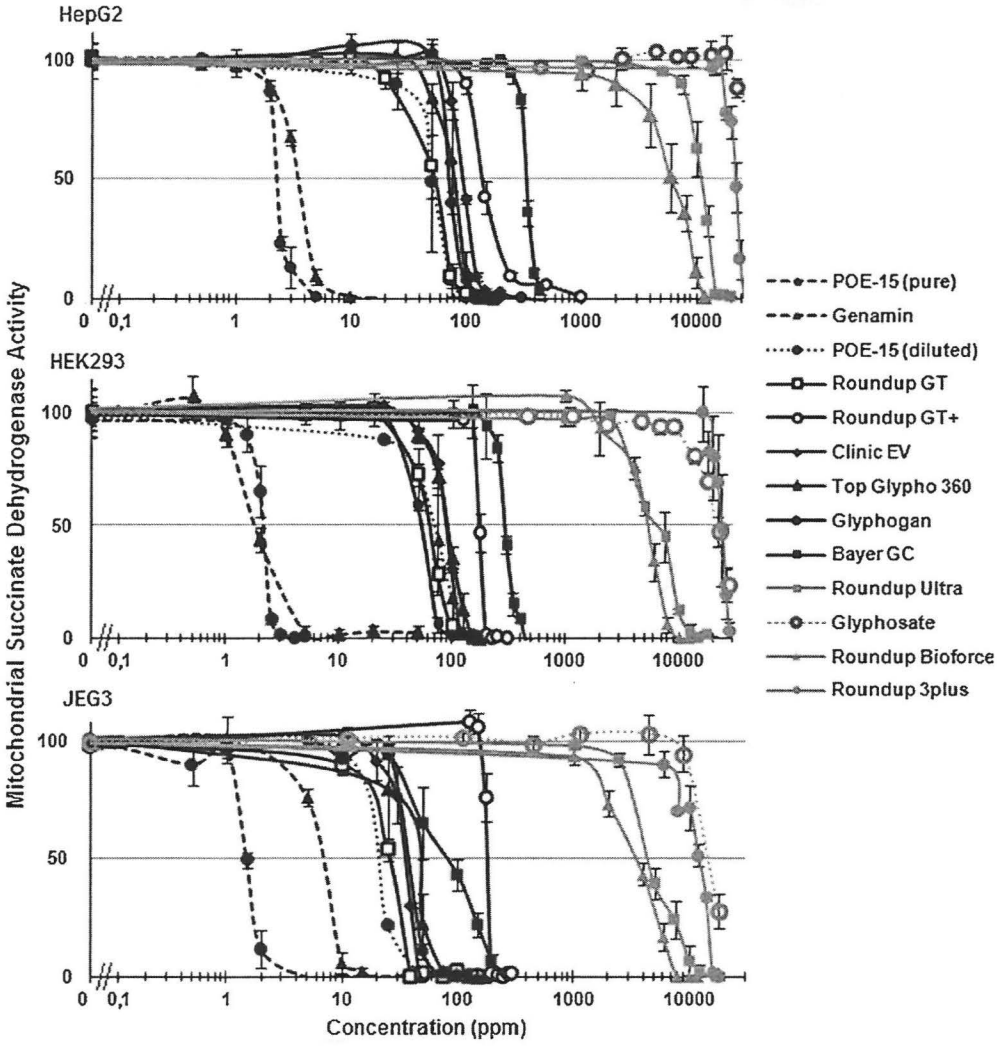

Fig. 2. Dose-dependent cytotoxic effects of glyphosate-based herbicides (GBH) or glyphosate (G) and adjuvants alone (POE-15 and Genamin) on HepG2, HEK293 and JEG3 human cell lines. Effects on the mitochondrial succinate dehydrogenase (SD) activity, reflecting cell respiration inhibition, were measured in % of control in serum-free medium after 24 h of exposure. The concentrations in ppm are dilutions of each mixture in the commercial formulation (considered as 100%). The adjuvants POE-15 and Genamin alone (a mixture containing 785 g/L of POE-15, no G) were the most toxic. The middle group approximately 100-fold less toxic was composed by GBH: Roundup GT, Roundup GT+, and Clinic EV, Top Glypho 360, Glyphogan, Bayer GC. The less toxic group was formed by Roundup Ultra, Bioforce and 3plus. SEMs are shown in all instances ($n = 9$).

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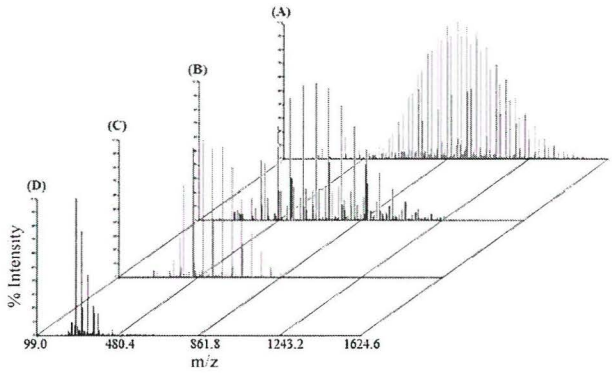

Fig. 3. MALDI-TOF analysis of glyphosate-based herbicides (GBH) main adjuvants. (A) POE-15 spectrum was centered on 900*m/z* (increment delta 44, Fig. 1), all other herbicides (group A, see Table 1) declaring a POEA adjuvant had the same spectrum. In addition, identification was confirmed by MS/MS fragmentation. (B) The 3 Roundup Ultra, Bioforce and 3plus contained another common adjuvant (600*m/z*, delta 58). (C) Adjuvants of Roundup GT+ (500*m/z*, delta 44, Fig. 1) were declared as ethoxylated etheralkylamines (EtO-EA). (D) Adjuvants of Roundup GT (300*m/z*, delta 44, Fig. 1) were identified as POE-2.

allowing the classification of the products tested as follows. The most toxic were the adjuvants alone POE-15 ($LC_{50} \sim 1$–2 ppm; agricultural dilutions: 1–2% of the herbicide formulation containing adjuvants) and Genamin, themselves around 100-fold more toxic than a middle group with the majority of formulations (6, with among them R GT and GT+). This middle group is again 100-fold more toxic than the third one which includes R Ultra, R Bioforce, R 3plus and finally G alone. Moreover, POE-15 diluted to the concentration at which it is present in Clinic E.V. (a formulation from the middle group) presented a similar toxicity than this GBH and to the middle group in general. It thus appears to be the toxic principle in human cells. In addition, we also demonstrate that two formulations claiming a similar concentration of G (360 g/L) and different adjuvants (16% of POEA or other adjuvants), Glyphogan and R Ultra respectively, exhibited very different toxicities, 150-fold stronger on average for Glyphogan on the 3 cell lines (Fig. 2). Thus some other adjuvants appear also to have some toxicity.

To check the composition in adjuvants we studied all the formulations by MALDI-TOF MS/MS (Fig. 3). Knowing that the specificities of MALDI-TOF ionization did not detect G but adjuvants, we separated 4 groups of adjuvants: (A) with a spectrum centered on 900*m/z*, POE-15 and Genamin, and those present in 4 formulations of the middle group thus containing also POE-15, (B) those contained in the third less toxic group with a spectrum centered on 600*m/z* corresponding to another common adjuvant, and (C) and (D), two other adjuvants in the formulations of the middle group, respectively in (C) R GT+ (500*m/z*) and (D) R GT (300*m/z*). The belonging of each product to each group was further confirmed by analysis of fragmentation spectra, giving for instance for ions of group A: 840.6, 858.7, 884.7, 902.8*m/z*. All these spectra corresponded to the family of alkylamines. The POE-15 had a peak increment of 44 (delta) like all group A (Table 1). The same delta in C and D were characteristic of an ethoxylated chain. C was an ethoxylated etheralkylamine, D was confirmed by fragmentation to be identical to POE-2; and a delta of 58 corresponded to another non ethoxylated adjuvant in group B. We summarized these findings with LC_{50} values (Table 1).

We then tested the linearity of the toxicity in function of G or ethoxylated adjuvants concentrations (Fig. 4). The cytotoxicity induced by GBH is not linear to G concentrations ($R^2 \sim 0.3$, Fig. 4A), but only to the 3 ethoxylated adjuvants ($R^2 > 0.93$, Fig. 4B), and not to the non-ethoxylated one, and this is obtained with all cell lines. Ethoxylated adjuvants can thus be considered as the active principle of the toxicity of GBH in human cells.

In order to understand the mechanism of action of adjuvants, three other experiments were performed. First, the critical micelle concentration (CMC) of POE-15 was determined by absorption changes of CBB R-250 (Fig. 5). The method was validated by the measurement of the CMC of the triton X-100 (0.15–0.20 mM (Courtney et al., 1986)). We evidenced a micellization of POE-15 beginning at 3 ppm, similarly to toxicity thresholds (Fig. 2). POE-15 thus appears to be able to disrupt the cellular membranes by micellization with the lipid bilayer around the CMC. This was even better understood by the differential measurement of the cytotoxicity through membrane disruption or caspases activation (Fig. 6). For the three cell lines, results are almost comparable: POE-15 and R GT+ (containing also an ethoxylated adjuvant) induced more necrosis (Fig. 6A) by membrane alterations rather than apoptosis (Fig. 6B), even if present. By contrast, G induced only apoptosis at higher levels. Ethoxylated adjuvants are thus not inert at all but cell membrane disruptors, and then induce severe mitochondrial alterations.

4. Discussion

This study unravels the differential nature and cytotoxicity of the main compounds from the major herbicide formulations in the world. These formulations are conceived to enhance the pesticide activity through mixtures of adjuvants and G. The latter is the active principle toxic in plants; in this study we checked how this

Table 1
Main spectral and toxicological characteristics of the herbicides (GBH) and adjuvants tested. Groups corresponded to spectra of adjuvants contained in products according to Fig. 3. Contents in glyphosate and adjuvants were indicated by manufacturers (except for POE-2) and identified by MS/MS as revealed by *m/z* and delta measurements. LC50 (ppm) are calculated from Fig. 2. nd: non detected.

Group	Products tested	Glyphosate (g/L)	Adjuvants	*m/z* (MS)	Delta (MS)	LC50 HepG2 (ppm)	LC50 HEK293 (ppm)	LC50 JEG3 (ppm)
A	Topglypho 360	360	15% POE-15	900	44	79	89	37
	Glyphogan	360	13–18% POE-15	900	44	59	54	30
	Clinic E.V	360	11% POE-15	900	44	94	89	34
	Bayer GC	96	1–5% POE-15	900	44	333	290	84
	Genamin	0	60–80% POE-15	900	44	4	2	7
	POE-15	0	POE-15	900	44	2	2	1
B	R Ultra	360	16% nc	600	58	11,000	6395	4477
	R Bioforce	360	nc	600	58	6106	5043	3560
	R 3plus	170	nc	600	58	22,000	24,000	1200
C	Roundup GT+	450	7.5% Eto-EA	500	44	145	170	115
D	Roundup GT	400	POE-2	300	44	53	62	32
	Glyphosate	>95%	nd	nd	nd	nd	19,323	1192

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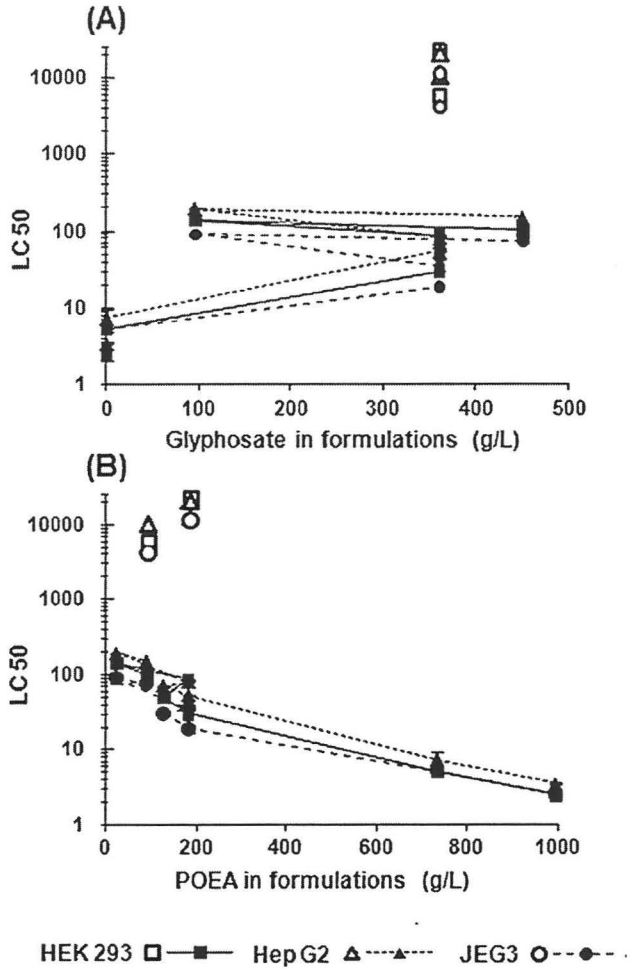

Fig. 4. Toxicity of glyphosate in formulations (A) measured by LC50, and of adjuvants in glyphosate-based herbicides (B) on the three human cell lines described. The effects on the mitochondrial succinate dehydrogenase (SD) activity were measured to calculate the LC50s (ppm) and compiled to be compared in relation to glyphosate or adjuvants concentrations. The form of the symbols is related to the cell lines (squares for HEK293, triangles for HepG2 and circles for JEG3). For colors, black dots are ethoxylated adjuvants, white dots are others. The three described human cell lines were used in the conditions of Fig. 2 and the results were almost identical. The linear correlation was not obtained (A) between glyphosate concentration and toxicity (coefficient of determination is 0.36 for HEK293, 0.35 for HepG2 and 0.29 for JEG3), but was demonstrated between the concentrations in the formulations of ethoxylated adjuvants (B) and toxicity (coefficient of determination is 0.94 for HEK293, 0.97 for HepG2 and 0.93 for JEG3). SEMs are represented in all instances ($n = 9$).

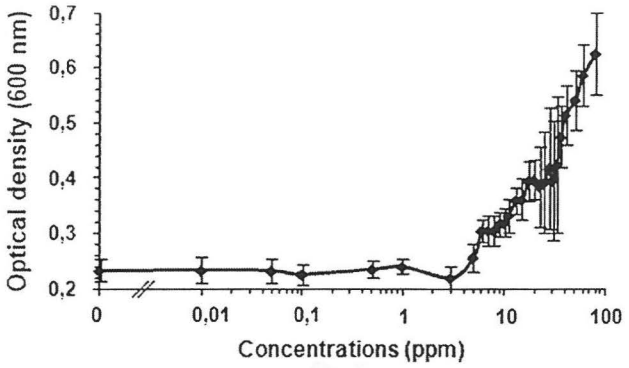

Fig. 5. Critical Micelle Concentration (CMC) of the POE-15 determined by absorption changes of Coomassie Brilliant Blue R-250. CBB R-250 was added to serial dilutions of POE-15 in serum-free medium. D.O. at 600 nm was measured with a spectrophotometer. A major breakpoint was evidenced in the curve around 3 ppm, at the CMC. SEMs are shown in all instances ($n = 9$).

active principle is differentially toxic on non-target organisms in comparison to the so-called inert adjuvants in numerous formulations.

Here we demonstrate that all formulations are more toxic than G alone on three human cell lines as previously underlined (Benachour and Seralini, 2009; Richard et al., 2005). Then for the first time we separated experimentally three groups of formulations differentially toxic according to the amount of ethoxylated adjuvants. The 3 less toxic formulations (like G alone) were demonstrated to contain no ethoxylated adjuvants by mass spectrometry, and are around 10,000 times less toxic on mitochondrial activity than POE-15 alone, the major adjuvant. All the other formulations were toxic proportionally to the dilutions of POE-15 or other ethoxylated adjuvants in the formulations, in a linear manner to some extent; in fact G does not buffer or amplify direct POE-15 toxicity.

Thus POE-15 appears to be clearly the toxic principle in human cells. It begins to be active with negative effects on cellular respiration and membrane integrity between 1 and 3 ppm, when its first micellization process occurs in this work. This membrane disruption then lead to the necrotic adjuvant-linked effects observed, amplifying the necrosis/apoptosis ratio by contrast to G at higher levels as shown. Accordingly, it was found (Chamel and Gambonnet, 1997) that a CMC of the $C_{18}NEO_{20}$ congener of a POEA is around 2 ppm. Its partition coefficient measured at around 1.7 confirmed its lipophilic character and its ability to penetrate the cells. It is known that ethoxylated adjuvants can insert in cells membranes, disrupting their structure and functions as previously shown in bacteria (Nobels et al., 2011). This is a general property of surfactants (Boeije et al., 2006). We notice that among different class of surfactants, ethoxylated adjuvants are of the more toxic, even potentially genotoxic (Nobels et al., 2011). Importantly, this is not only observed in vitro because when rats are treated with G, R and POEA, the latter was also found to be the most toxic compound (Adam et al., 1997), even in other animal models (Marc et al., 2005). This was demonstrated for other pesticides (Eddleston et al., 2012). Generally, the question of the toxicity of adjuvants in pesticides is more and more recognized (Brausch and Smith, 2007; Krogh et al., 2003; Tsui and Chu, 2003).

This does not exclude cellular endocrine disruptions below these levels that may not be due to POE-15 alone (or other ethoxylated adjuvants), but that occur through glyphosate entering in aromatase active site for instance (Richard et al., 2005) or in androgen receptor which is inhibited from 0.2 ppm of G in adjuvants (Gasnier et al., 2009). It should not be forgotten that G has its own toxicity and may also exert long term or chronic toxicity. The active principle G alone has been evidenced to cause oxidative stress (Astiz et al., 2009; Cavusoglu et al., 2011), endocrine disruption (Clair et al., 2012), or developmental effects (Marc et al., 2005). G was even recently described as a teratogen (Paganelli et al., 2010). In this case we have a model of multiple combined negative effects (through different cellular metabolic endpoints) caused by the main pesticide mixtures, which are the formulations themselves. This is true even if the activities of ethoxylated adjuvants on endocrine disruption must be still detailed in the future.

These results were obtained in vitro; cellular cultures replace whenever it is possible animal experimentation (Hartung, 2009). Our study was performed during 24 h and does not anticipate the elimination or the possible bioaccumulation and long term combined effects with other xenobiotics. R human cellular effects indeed increased according to time (Benachour et al., 2007) and radiolabeled G accumulated in cells within 48 h, suggesting a

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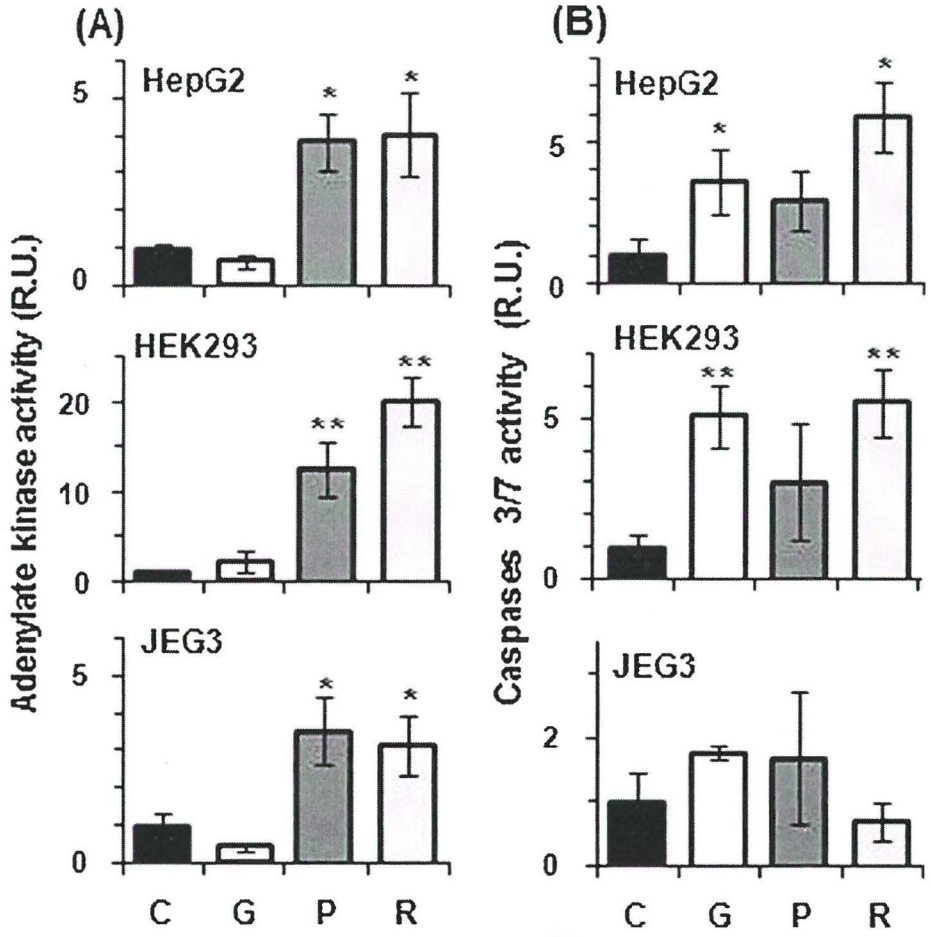

Fig. 6. Cytotoxic effects of control (C), glyphosate (G), POE-15 (P) and Roundup GT+ (R). Cell membrane integrity reflecting necrosis (A) was measured by adenylate kinase leakage (active in the medium), and apoptosis (B) by caspases 3/7 activities, both expressed in relative units (RU) after 24 h of treatments like in Fig. 2. To understand the mechanism of cytotoxicity, the concentrations in products were those inducing 80% of the general cytotoxicity in MTT assay. SEMs are shown in all instances ($n = 12$, $^{*}p < 0.05$; $^{**}p < 0.01$).

bioaccumulation of low concentrations of G (Gasnier et al., 2011). R adjuvants may also form adducts and link to DNA avoiding a direct elimination (Peluso et al., 1998).

However, our lowest thresholds of toxicities and endocrine disruptions may be comparable to the range of environmental/occupational exposures. A farmer or a gardener spraying a GBH may be punctually exposed to 5000 ppm, and even regularly by occupational exposure. As a matter of fact G varied from 3 to 233 ppb in farmers urine (Acquavella et al., 2004), this may be in addition to a chronic dietary/drink exposure of G found around 70 ppb (Aris and Leblanc, 2011).

In conclusion, pesticide formulations should be studied as mixtures for toxic effects. The multiple combined effects could induce pathologies on a long term. Here we can question the use of ethoxylated adjuvants in herbicide formulations, since they appear as principles for human cell toxicity. This leads also to challenge guidance values such as the acceptable daily intake (ADI) of G, which is calculated with pure G in long term toxicological tests in vivo (German Federal Agency CPFS, 1998), while G is always used with adjuvants that are not immediately biodegradable (Banduhn and Frazier, 1978) and could change its toxicity. This will be also important for other active principles of pesticides, and thus their ADI can be overestimated. The necessity of studying formulations as mixtures is common to all pesticides. The pathological consequences of exposure to chronic toxicities of whole formulations could be tested with mammals over a 2-year period. This implies a complete shift in the concepts underlying chemical toxicology, which could come from mixtures studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

Acquavella, J.F., Alexander, B.H., Mandel, J.S., Gustin, C., Baker, B., Chapman, P., Bleeke, M., 2004. Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. Environ. Health Perspect. 112, 321–326.

Adam, A., Marzuki, A., Abdul Rahman, H., Abdul Aziz, M., 1997. The oral and intratracheal toxicities of ROUNDUP and its components to rats. Vet. Hum. Toxicol. 39, 147–151.

Aris, A., Leblanc, S., 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. Reprod. Toxicol. 31, 528–533.

Astiz, M., de Alaniz, M.J., Marra, C.A., 2009. Effect of pesticides on cell survival in liver and brain rat tissues. Ecotoxicol. Environ. Saf. 72, 2025–2032.

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Banduhn, M., Frazier, H., 1978. G 3780A surfactant: biodegradation in nature waters. Report No. MSL-0488. Monsanto Agricultural Research Department, St. Louis.
Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Seralini, G.E., 2007. Time- and dose-dependent effects of roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53, 126–133.
Benachour, N., Seralini, G.E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22, 97–105.
Beuret, C.J., Zirulnik, F., Gimenez, M.S., 2005. Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. Reprod. Toxicol. 19, 501–504.
Boeije, G.M., Cano, M.L., Marshall, S.J., Belanger, S.E., Van Compernolle, R., Dorn, P.B., Gumbel, H., Toy, R., Wind, T., 2006. Ecotoxicity quantitative structure–activity relationships for alcohol ethoxylate mixtures based on substance-specific toxicity predictions. Ecotoxicol. Environ. Saf. 64, 75–84.
Boocock, M.R., Coggins, J.R., 1983. Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. FEBS Lett. 154, 127–133.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.
Brausch, J.M., Smith, P.N., 2007. Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, *Thamnocephalus platyurus*. Arch. Environ. Contam. Toxicol. 52, 217–221.
Cavusoglu, K., Yapar, K., Oruc, E., Yalcin, E., 2011. Protective effect of *Ginkgo biloba* L. leaf extract against glyphosate toxicity in Swiss albino mice. J. Med. Food 14, 1263–1272.
Chamel, A., Gambonnet, B., 1997. Sorption and diffusion of an ethoxylated stearic alcohol and an ethoxylated stearic amine into and through isolated plant cuticles. Chemosphere 34, 1777–1786.
Clair, E., Mesnage, R., Travert, C., Seralini, G.E., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. In Vitro 26, 269–279.
Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.
Corbera, M., Simonet, B.M., Salvado, V., Hidalgo, M., 2010. Characterisation of alkylamine ethoxylates (ANEOs) in commercial herbicide formulations using liquid chromatography/electrospray ionisation mass spectrometry. Rapid Commun. Mass Spectrom. 24, 2931–2937.
Courtney, H.S., Simpson, W.A., Beachey, E.H., 1986. Relationship of critical micelle concentrations of bacterial lipoteichoic acids to biological activities. Infect. Immun. 51, 414–418.
Cox, C., 2004. Herbicide factsheet—glyphosate. J. Pesticide Reform 24, 10–15.
Crouch, S.P., Kozlowski, R., Slater, K.J., Fletcher, J., 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J. Immunol. Methods 160, 81–88.
Cserháti, T., Forgács, E., 1997. Separation and quantitative determination of non-ionic surfactants used as pesticide additives. J. Chromatogr. A 774, 265–279.
Dallegrave, E., Mantese, F.D., Oliveira, R.T., Andrade, A.J., Dalsenter, P.R., Langeloh, A., 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch. Toxicol. 81, 665–673.
Daruich, J., Zirulnik, F., Gimenez, M.S., 2001. Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. Environ. Res. 85, 226–231.
Eddleston, M., Street, J.M., Self, I., Thompson, A., King, T., Williams, N., Naredo, G., Dissanayake, K., Yu, L.M., Worek, F., John, H., Smith, S., Thiermann, H., Harris, J.B., Eddie Clutton, R., 2012. A role for solvents in the toxicity of agricultural organophosphorus pesticides. Toxicology 294, 94–103.
Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C., Seralini, G.E., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262, 184–191.
Gasnier, C., Laurant, C., Decroix-Laporte, C., Mesnage, R., Clair, E., Travert, C., Seralini, G.E., 2011. Defined plant extracts can protect human cells against combined xenobiotic effects. J. Occup. Med. Toxicol. 6, 3.
German Federal Agency CPFS, 1998. Monograph on Glyphosate. Released by the German Federal Agency for Consumer Protection and Food Safety. Annex B-5: Toxicology and Metabolism, p. 136.
Hartung, T., 2009. Toxicology for the twenty-first century. Nature 460, 208–212.
Hawthorne, D.J., Dively, G.P., 2011. Killing them with kindness? In-hive medications may inhibit xenobiotic efflux transporters and endanger honey bees. PLoS ONE 6, e26796.
IFEN, 2006. Report on Pesticides in Waters. Data 2003–2004.
James, C., 2011. Global Status of Commercialized Biotech/GM Crops: 2009. ISAAA Brief 43.
Krogh, K.A., Halling-Sorensen, B., Mogensen, B.B., Vejrup, K.V., 2003. Environmental properties and effects of nonionic surfactant adjuvants in pesticides: a review. Chemosphere 50, 871–901.
L'Azou, B., Fernandez, P., Bareille, R., Beneteau, M., Bourget, C., Cambar, J., Bordenave, L., 2005. In vitro endothelial cell susceptibility to xenobiotics: comparison of three cell types. Cell Biol. Toxicol. 21, 127–137.
Letcher, R.J., van Holsteijn, I., Drenth, H.J., Norstrom, R.J., Bergman, A., Safe, S., Pieters, R., van den Berg, M., 1999. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxicol. Appl. Pharmacol. 160, 10–20.
Liu, J.J., Wang, W., Dicker, D.T., El-Deiry, W.S., 2005. Bioluminescent imaging of TRAIL-induced apoptosis through detection of caspase activation following cleavage of DEVD-aminoluciferin. Cancer Biol. Ther. 4, 885–892.
Marc, J., Le Breton, M., Cormier, P., Morales, J., Belle, R., Mulner-Lorillon, O., 2005. A glyphosate-based pesticide impinges on transcription. Toxicol. Appl. Pharmacol. 203, 1–8.
Mesnage, R., Clair, E., Séralini, G.E., 2010. Roundup in genetically modified plants: regulation and toxicity in mammals. Theor. Ökol. 16, 31–33.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Nobels, I., Spanoghe, P., Haesaert, G., Robbens, J., Blust, R., 2011. Toxicity ranking and toxic mode of action evaluation of commonly used agricultural adjuvants on the basis of bacterial gene expression profiles. PLoS ONE 6, e24139.
Oliveira, A.G., Telles, L.F., Hess, R.A., Mahecha, G.A., Oliveira, C.A., 2007. Effects of the herbicide Roundup on the epididymal region of drakes *Anas platyrhynchos*. Reprod. Toxicol. 23, 182–191.
Paganelli, A., Gnazzo, V., Acosta, H., Lopez, S.L., Carrasco, A.E., 2010. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem. Res. Toxicol. 23, 1586–1595.
Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115–1122.
Peluso, M., Munnia, A., Bolognesi, C., Parodi, S., 1998. 32P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. Environ. Mol. Mutagen. 31, 55–59.
Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720.
Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., de Souza, P.B., Viau, P., Bernardi, M.M., Nunes, M.T., de Oliveira, C.A., 2011. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch. Toxicol. 86, 663–673.
Samsonoff, C., Daily, J., Almog, R., Berns, D.S., 1986. The use of coomassie brilliant blue for critical micelle concentration determination of detergents. J. Colloid Interface Sci. 109, 325–329.
Savitz, D.A., Arbuckle, T., Kaczor, D., Curtis, K.M., 1997. Male pesticide exposure and pregnancy outcome. Am. J. Epidemiol. 146, 1025–1036.
Tsui, M.T., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197.
Urani, C., Doldi, M., Crippa, S., Camatini, M., 1998. Human-derived cell lines to study xenobiotic metabolism. Chemosphere 37, 2785–2795.
Williams, A.L., Watson, R.E., Desesso, J.M., 2012. Developmental and reproductive outcomes in humans and animals after glyphosate exposure: a critical analysis. J. Toxicol. Environ. Health B: Crit. Rev. 15, 39–96.
Yousef, M.I., Salem, M.H., Ibrahim, H.Z., Helmi, S., Seehy, M.A., Bertheussen, K., 1995. Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. J. Environ. Sci. Health B 30, 513–534.

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Research Article

Major Pesticides Are More Toxic to Human Cells Than Their Declared Active Principles

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Pesticides are used throughout the world as mixtures called formulations. They contain adjuvants, which are often kept confidential and are called inerts by the manufacturing companies, plus a declared active principle, which is usually tested alone. We tested the toxicity of 9 pesticides, comparing active principles and their formulations, on three human cell lines (HepG2, HEK293, and JEG3). Glyphosate, isoproturon, fluroxypyr, pirimicarb, imidacloprid, acetamiprid, tebuconazole, epoxiconazole, and prochloraz constitute, respectively, the active principles of 3 major herbicides, 3 insecticides, and 3 fungicides. We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. Fungicides were the most toxic from concentrations 300–600 times lower than agricultural dilutions, followed by herbicides and then insecticides, with very similar profiles in all cell types. Despite its relatively benign reputation, Roundup was among the most toxic herbicides and insecticides tested. Most importantly, 8 formulations out of 9 were up to one thousand times more toxic than their active principles. Our results challenge the relevance of the acceptable daily intake for pesticides because this norm is calculated from the toxicity of the active principle alone. Chronic tests on pesticides may not reflect relevant environmental exposures if only one ingredient of these mixtures is tested alone.

1. Introduction

Pesticides are used throughout the world as mixtures called formulations. They contain adjuvants, which are often kept confidential and are called inerts by the manufacturing companies, plus a declared active principle (AP), which is the only one tested in the longest toxicological regulatory tests performed on mammals. This allows the calculation of the acceptable daily intake (ADI)—the level of exposure that is claimed to be safe for humans over the long term—and justifies the presence of residues of these pesticides at "admissible" levels in the environment and organisms. Only the AP and one metabolite are used as markers, but this does not exclude the presence of adjuvants, which are cell penetrants. Our previous investigation showed unexpected APs for human cell toxicity in the adjuvants of glyphosate-based herbicides [1]. Ethoxylated adjuvants found in glyphosate-based herbicides were up to 10.000 times more toxic than the so-called active AP glyphosate [1] and are better candidates for secondary side effects. This may explain in vivo long-term toxicity from 0.1 ppb of the formulation and other toxicities that were not explained by a consideration of glyphosate alone [2–5]. These adjuvants also have serious consequences to the health of humans and rats in acute exposures [6, 7]. These findings prompted us to investigate the presence of similar toxic molecules in other classes of pesticides.

The regulatory system assumes that the AP designed to specifically target plants, insects or fungi is the most toxic compound of a formulation to nontarget species. Thus long-term regulatory tests are performed on this substance alone. In this paper, we tested to what extent the AP or adjuvants in present formulations account for the toxicity of 9 major pesticides: 3 herbicides, 3 insecticides, and 3 fungicides.

We have thus selected 9 APs of herbicides, insecticides, or fungicides of different classes (Table 1) used for agricultural or domestic purposes, from the major pesticides

Table 1: Summary of the pesticides tested. We have tested 9 APs of major herbicides, insecticides, or fungicides of different classes, used worldwide for agricultural or domestic purposes. Concentrations of the APs are indicated in parenthesis. Adjuvants are reported where they are mentioned on the material safety data sheet (MSDS).

	Pesticide classes	Active principles	(g/L)	Formulations	Declared adjuvants
Herbicides	Phosphonoglycine	Glyphosate	450	Roundup GT+	Ethoxylated etheralkylamine
	Phenylurea	Isoproturon	500	Matin EL	Unknown
	Synthetic auxin	Fluroxypyr (ester 1-methylheptyl)	200	Starane 200	Solvent naphtha; alkyl-aryl sulfonates
Insecticides	Carbamate	Pirimicarb	500	Pirimor G	Docusate sodium; benzenesulfonic acid
	Neonicotinoid	Imidacloprid	200	Confidor	1-Methyl-2-pyrrolidinone
	Neonicotinoid	Acetamiprid	5	Polysect Ultra	1,2-Benzisothiazoline-3-one; ethanol
Fungicides	Triazole	Tebuconazole	250	Maronee	N,N-Dimethyldecanamide
	Triazole	Epoxiconazole	125	Opus	Solvent naphtha; fatty alcohol ethoxylated
	Imidazole	Prochloraz	450	Eyetak	Solvent naphtha; xylene; isobutanol

used worldwide [8, 9]. First we tested Roundup and its AP, glyphosate. Upon the introduction of herbicide tolerant genetically modified organisms (GMOs), designed to tolerate Roundup and to accumulate unusual levels of its residues, Roundup quickly became the major pesticide in the world and a major food or feed contaminant [10]. Two other herbicides of a different class were tested: isoproturon (phenylurea) is the second most widely used AP of herbicides in Europe in the control of annual grasses and broad-leaved weeds in cereals and a major water contaminant [11]; and fluroxypyr (a synthetic auxin) is used as an AP on noncrop areas and also for agricultural use on wheat, barley, corn, and oats. Forest services are expanding its use as an alternative to other pesticides known to be toxic [12]. However, it is poorly studied and its effects on human cells were never published before. Among the insecticides chosen, pirimicarb (a carbamate), used specifically to target aphids, is the most representative AP in this family for cereal production and garden insect control worldwide [13]. Neonicotinoids are the largest selling insecticides worldwide and are marketed in more than 120 countries for use on more than 140 crops [14]. Their spectrum of biological efficacy covers a broad range of target pests such as whiteflies, lepidopteran, and coleopteran species. We tested the major neonicotinoid, the AP imidacloprid, which is widely used for seed dressing. Its toxicity against bees is widely admitted [15], but little is known about the effects of its adjuvants. We also tested the AP acetamiprid, another neonicotinoid advocated to replace imidacloprid [16]. Azole-type fungicides are applied every year on field crops, fruit trees, vegetables, and grassgrowing areas [17]. We tested the two most popular triazole APs, epoxiconazole and tebuconazole. Finally, prochloraz (imidazole) was tested because it is the main fungicide sprayed on cereals in Europe [8].

We used the embryonic (HEK293), placental (JEG3), and hepatic (HepG2) human cell lines because they are well characterized and validated as useful models to test toxicities of pesticides [18–20], corresponding to what is observed on fresh tissue or primary cells [21–23]. These cell lines are even in some instances less sensitive than primary cells [24, 25] and therefore do not overestimate cellular toxicity. We assayed their mitochondrial succinate dehydrogenase (SD) activity (MTT assay) after 24 h pesticide exposure, which is one of the most accurate cytotoxicity assays for measuring the toxicity of pesticide adjuvants such as surfactants [26]. Cytotoxicity was confirmed by the measurement of apoptosis and necrosis, respectively, by caspases 3/7 activation [27] and adenylate kinase leakage after membrane alterations [28]. Each AP was tested from levels below its ADI to its solubility limit in our system. The formulations containing adjuvants were tested at the same levels.

2. Materials and Methods

2.1. Chemicals. The 9 Aps, glyphosate (N-phosphonomethyl glycine, G, CAS: 1071-83-6), isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea, CAS: 34123-59-6), fluroxypyr 1-methylheptyl ester (((4-Amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy)acetic acid, 1-methylheptyl ester, CAS: 81406-37-3), acetamiprid (N-[(6-chloro-3-pyridyl) methyl]-N′-cyano-N-methyl-acetamidine, CAS: 135410-20-7), imidacloprid (1-((6-chloro-3-pyridinyl)methyl)-4,5-dihydro-N-nitro-1H-imidazol-2-amine, CAS: 105827-78-9), pirimicarb (2-dimethylamino-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate, CAS: 23103-98-2), prochloraz (N-propyl-N-(2,4,6-trichlorophenoxy) ethyl-imidazole-1-carboxamide, CAS: 67747-09-5), epoxiconazole (1-{[3-(2-Chlorophenyl)-2-(4-fluorophenyl)-2-oxiranyl]methyl}-1H-1,2,4-triazole, CAS: 135319-73-2), tebuconazole (1-(4-Chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazole-1-ylmethyl)pentane-3-ol, CAS: 107534-96-3), and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as well as all other compounds, unless otherwise noted, were obtained from Sigma-Aldrich. Formulations were available on the market: Roundup GT+ (approval 2020448), Matin EL (2020328), Starane 200 (8400600), Pirimor G (7500569), Confidor (9200543), Polysect Ultra SL (2080018), Maronee (2000420), Opus (9200018), and Eyetak (9400555). MTT was prepared as a 5 mg/mL stock solution in phosphate-buffered saline, filtered through a 0.22 μm filter before use, and diluted to 1 mg/mL in a serum-free medium.

2.2. Cell Lines and Treatments. The human embryonic kidney 293 cell line (HEK 293, ECACC 85120602) was provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). The hepatoma cell line HepG2 was provided by ECACC

(85011430). JEG3 cell line (ECACC 92120308) was provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM (Abcys, Paris, France) containing 2 mM glutamine, 1% nonessential amino acid, 100 U/mL of antibiotics (a mixture of penicillin, streptomycin, and fungizone) (Lonza, Saint Beauzire, France), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France), and 10% Fetal Bovine Serum (PAA, les Mureaux, France). JEG3 cells were supplemented with 1 mM sodium pyruvate. Cells were grown with this medium at 37°C (5% CO_2, 95% air) during 48 h to 80% confluence, then washed, and exposed 24 h with serum-free EMEM to the APs or the formulations. Before treatment, all the pesticides were solubilized in a 100% DMSO solution, then diluted in serum-free medium to reach 0.5% DMSO (which had been previously proven not to be cytotoxic for the cells), and adjusted to a similar pH. This model was validated [29] and cytotoxic effects were similar in presence of serum but delayed by 48 h.

2.3. Cytotoxicity Measurement. After treatments, succinate dehydrogenase (SD) activity assay (MTT) [30] was applied as described previously [25]. Integrity of mitochondrial dehydrogenase enzymes indirectly reflects the cellular mitochondrial respiration. The optical density was measured at 570 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The bioluminescent ToxiLight bioassay (Lonza, Saint Beauzire, France) was applied for the membrane degradation assessment, by the intracellular adenylate kinase (AK) release in the medium; this is described as a necrosis marker [28]. Finally, the apoptotic cell death was evaluated with the Caspase-Glo 3/7 assay (Promega, Paris, France). Luminescence was measured using a Mithras LB 940 luminometer (Berthold, Thoiry, France). These methods were previously described [25].

2.4. Statistical Analysis. The experiments were repeated at least 3 times in different weeks on 3 independent cultures ($n = 9$). All data were presented as the means ± standard errors (SEMs). LC50 values were the best-fitted value of a nonlinear regression using sigmoid (5-parameter) equation with the GraphPad Prism 5 software. The differential effects between APs and formulations are measured by the surfaces between the curves by the calculation of integrals with ImageJ software [31]. Statistical differences of necrosis and apoptosis assays were calculated by a nonparametric Mann-Whitney test with the GraphPad Prism 5 software.

3. Results

All formulations were cytotoxic and far more toxic than their APs, except for isoproturon and its formulated pesticide Matin which were both not soluble over 100 ppm. As a matter of fact, Matin does not have any declared adjuvant (Table 1). On human cells, among the tested products, fungicides were the most toxic (Figure 1), being cytotoxic from doses 300–600 times lower than agricultural dilutions, followed by herbicides (Figure 2) (except Matin) and then insecticides (Figure 3). JEG3 was the most sensitive cell line, the LC50 being on average, respectively, 7% and 23% lower than for HEK293 and HepG2, the least sensitive. The LC50 is calculated over 24 h. In all cell types, fungicides were the most toxic (mean LC50 12 ppm). They were followed by the herbicide Roundup (LC50 63 ppm), twice as toxic as Starane, and more than 10 times as toxic as the 3 insecticides, which represent the less toxic group (mean LC50 720 ppm). The APs of fungicides were the only APs that were toxic alone in our system, from 50 ppm in JEG3 for prochloraz, but they were still less toxic than their formulations.

In fact, 8 formulations out of 9 were clearly on average several hundred times more toxic than their APs, ranging from 2-3 times more toxic for pirimicarb or prochloraz to 1056 times more toxic for tebuconazole. Results were similar for all cell types.

This was even better understood by the differential measurement of the cytotoxicity through membrane disruption (Figure 4) or caspases activation (Figure 5). For the three cell lines, membrane disruptions are comparable. Most of the pesticides were necrotic and more necrotic than their APs except for Eyetak whose active principle prochloraz is the main toxicant of the formulation. We have not obtained relevant results with Pirimor because a green dye in the formulated product prevents the lecture of luminescence. Differential effects on apoptosis (Figure 5) were less obvious. With the formulated herbicides and insecticides, apoptosis levels are mostly decreased because of the prevailing effects of necrosis. This is not the case with fungicides which are apoptotic depending on the cell line. JEG3 cell lines are the most sensitive to apoptosis, in particular with fluroxypyr, pirimicarb, tebuconazole, and prochloraz. Overall, adjuvants in pesticides are thus far from inerts but cell membrane disruptors and induce in addition mitochondrial alterations.

4. Discussion

This is the first time that all these formulated pesticides have been tested on human cells well below agricultural dilutions. The three different cell types reacted very similarly and the toxicities were observed on several biomarkers; this confirmed our results. Moreover, these are very consistent with several studies on cell lines [1, 25], where placental JEG3 cells were found to be the most sensitive. In this study [1], adjuvants were also more cytotoxic through the disruption of membrane and mitochondrial respiration than from an activation of apoptotic pathways. Primary cells are in some case up to 100 times more sensitive, for instance, neonate umbilical cord vein cells [25]. We also study here short exposures (24 h), but we have previously demonstrated a time-amplifying effect: the differential toxicity between the AP glyphosate and Roundup is increased by 5 times in 72 h [29]. It appears that, with cell lines and short exposures, we underestimate by far the direct toxicity of the products in the long term. In this case in vivo, the metabolism may reduce the toxic effect, but this can be compensated or amplified by bioaccumulation and/or the combined effect of the AP with adjuvants. For instance, in this experiment, after 24 h, 63 ppm of Roundup was found to be toxic to cells, but in our previous

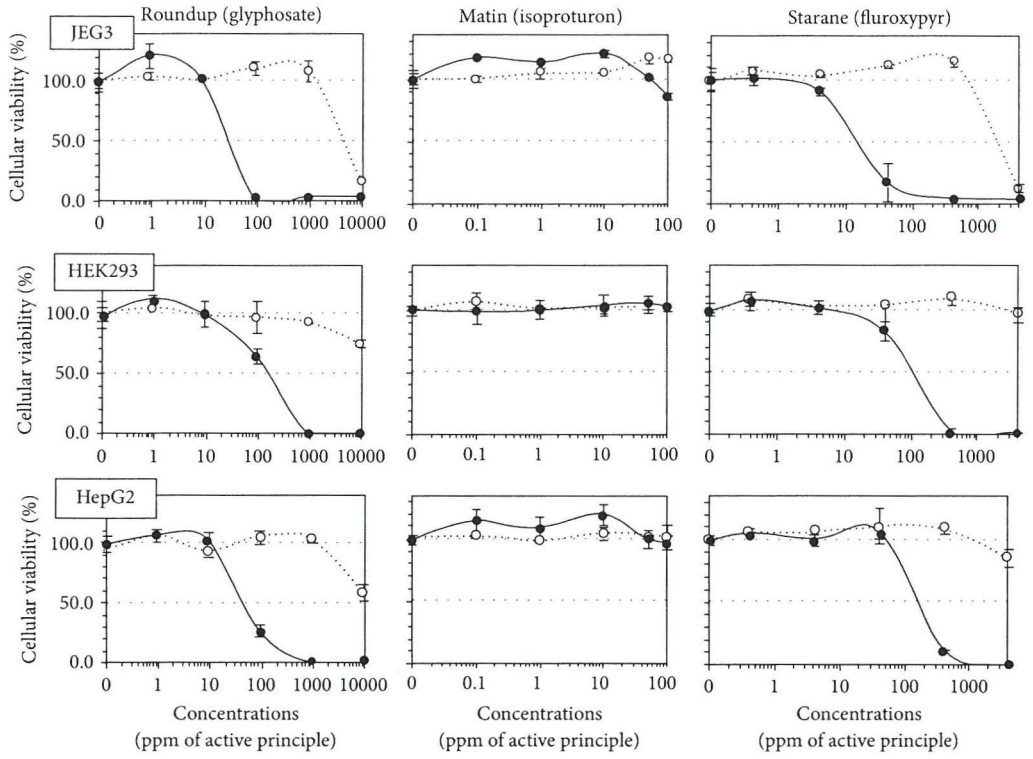

FIGURE 1: Differential cytotoxic effects between formulations of herbicides and their active principles (APs) on HepG2, HEK293, and JEG3 human cell lines. Effects on the mitochondrial succinate dehydrogenase (SD) activity, reflecting cell respiration inhibition, were measured in percentage of control in serum-free medium after 24 h of exposure. The concentrations in ppm are dilutions of each AP (dotted line) and their equivalent in formulation with adjuvants (solid line). All formulations are more toxic than their APs, except for isoproturon. SEMs are shown in all instances ($n = 9$).

experiment, after two years in rats, only 0.1 ppb of Roundup was found to be sufficient to provoke pathologies [2].

Adjuvants in pesticides are generally declared as inerts, and for this reason they are not tested in long-term regulatory experiments. It is thus very surprising that they amplify up to 1000 times the toxicity of their APs in 100% of the cases where they are indicated to be present by the manufacturer (Table 1). In fact, the differential toxicity between formulations of pesticides and their APs now appears to be a general feature of pesticides toxicology. As we have seen, the role of adjuvants is to increase AP solubility and to protect it from degradation, increasing its half-life, helping cell penetration, and thus enhancing its pesticidal activity [32] and consequently side effects. They can even add their own toxicity [1]. The definition of adjuvants as "inerts" is thus nonsense; even if the US Environmental Protection Agency has recently changed the appellation for "other ingredients," pesticide adjuvants should be considered as toxic "active" compounds.

In the scientific literature, in contrast with regulatory beliefs, some harmful effects of the adjuvants present in this study are reported. In the formulations (Table 1) Starane 200, Opus, and Eyetak, the adjuvants include solvent naphtha (a petroleum distillate), which is known to have developmental effects in rodents [33]. Xylene (in Eyetak) has long been associated with cardiac and central nervous system diseases in humans [34]. 1-Methyl-2-pyrrolidinone (in Confidor) is a developmental toxicant and caused malformations, incomplete ossification of skull, and decreased fetal body weights in rats [35]. N,N-Dimethyldecanamide (Maronee adjuvant) has been characterized as a developmental toxicant in rodents [36] but is insufficiently studied for reproductive toxicity. The distinction between AP and "declared inert" compounds appears to be a regulatory assumption with no toxicological basis, from this experiment and others. Even industry and regulators contradict themselves in the classification of APs and inert compounds. For example, 1,2-benzisothiazoline-3-one is classed as an inert ingredient in the pesticide Polysect in particular and as an active ingredient in cleaning products [37].

All this does not exclude the toxicity of APs alone. Glyphosate inserted in the aromatase active site of mammalian cells disrupts steroidogenesis [23]. Imidacloprid alters the developing immunity in rats [38]. Fluroxypyr (ester 1-methylheptyl) has never been tested in human cells before this study but appears to be toxic from 22 ppm in formulation; its ADI is only 0.8 ppm/day (DG SANCO, 2013). It also appears here that prochloraz is the main toxicant of the tested formulation.

It is commonly believed that Roundup is among the safest pesticides. This idea is spread by manufacturers, mostly in the reviews they promote [39, 40], which are often cited

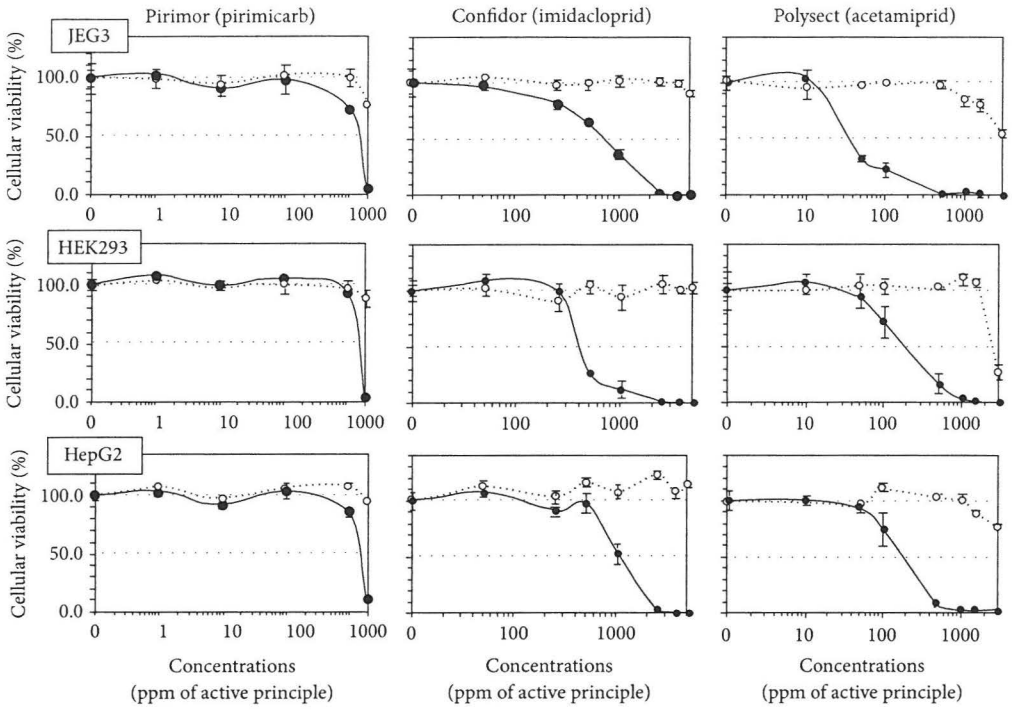

FIGURE 2: Differential cytotoxic effects between formulations of insecticides and their APs on HepG2, HEK293, and JEG3 human cell lines. The three described human cell lines were used in the conditions of Figure 1 and the results were almost identical. All formulations (solid line) are more toxic than their APs (dotted line); APs are slightly cytotoxic. SEMs are shown in all instances ($n = 9$).

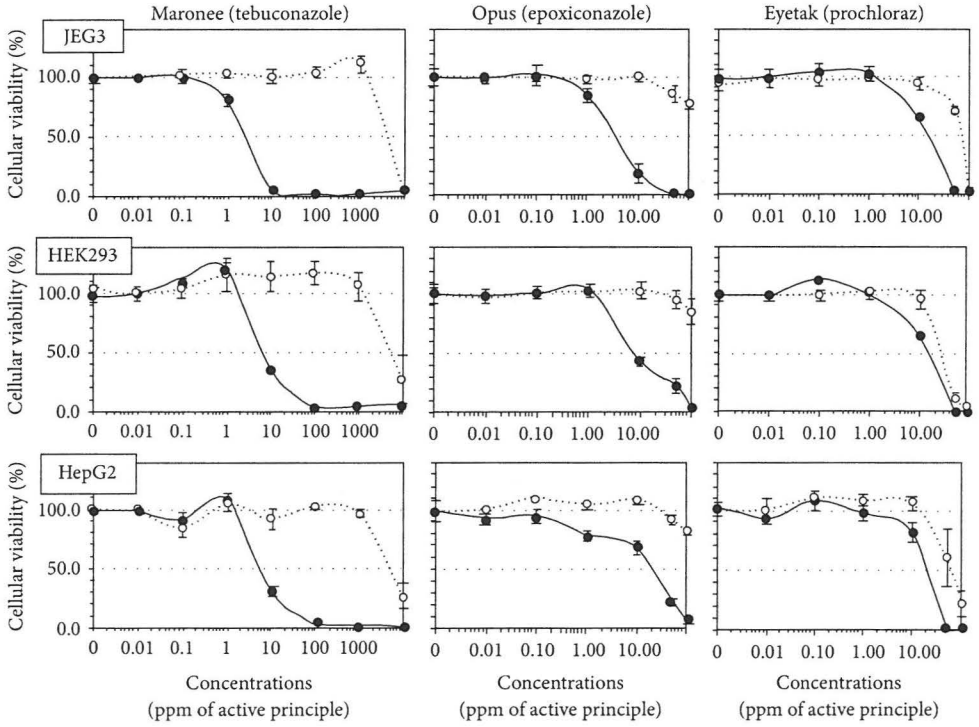

FIGURE 3: Differential cytotoxic effects between formulations of fungicides and their APs on HepG2, HEK293, and JEG3 human cell lines. The three described human cell lines were used in the culture conditions of Figure 1, and the results were almost identical. All formulations (solid line) are more cytotoxic than their APs (dotted line). Maronee is the most toxic compound tested from 1 ppm in JEG3. SEMs are shown in all instances ($n = 9$).

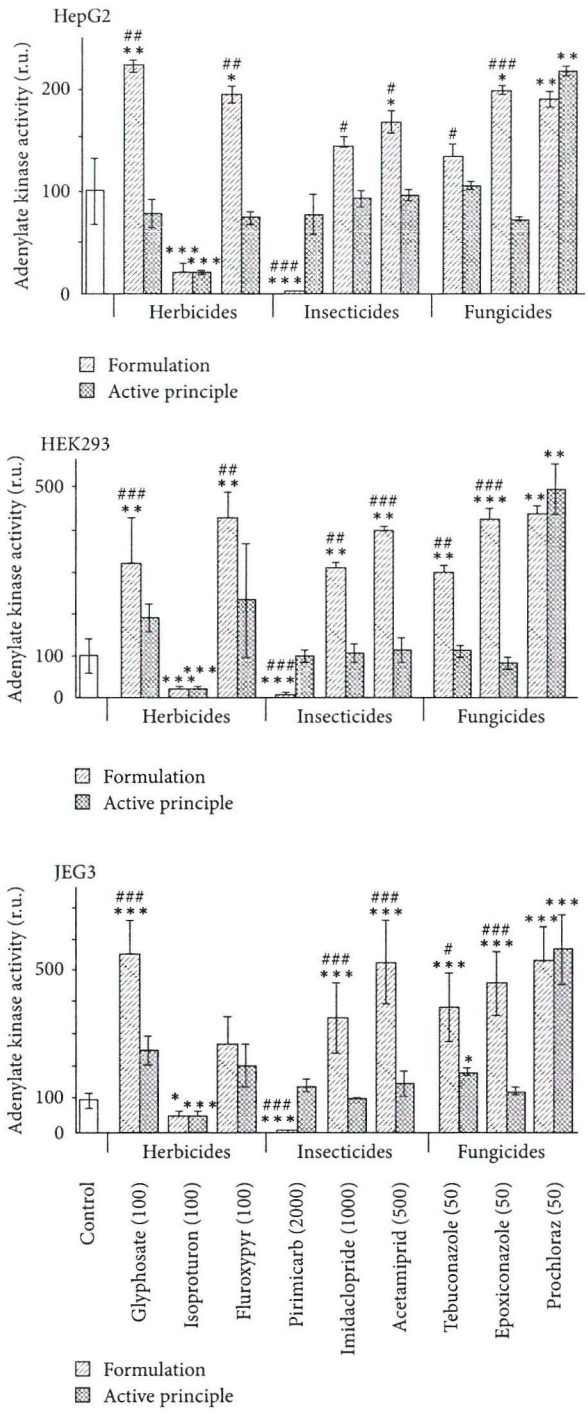

FIGURE 4: Differential necrotic effects between formulations and their APs. The three described human cell lines were used in the culture conditions of Figure 1. We have chosen the doses at the first differential effects measured by MTT assay. Formulations (stripped columns, expressed in ppm of the AP) are generally more cytotoxic than their APs (dashed columns) due to a necrotic effect of adjuvants. SEMs are shown in all instances ($n = 9$). For the comparison of each AP or formulation to the control (white column), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in a nonparametric Mann-Whitney test. # symbol is used similarly for comparisons between APs and their formulations.

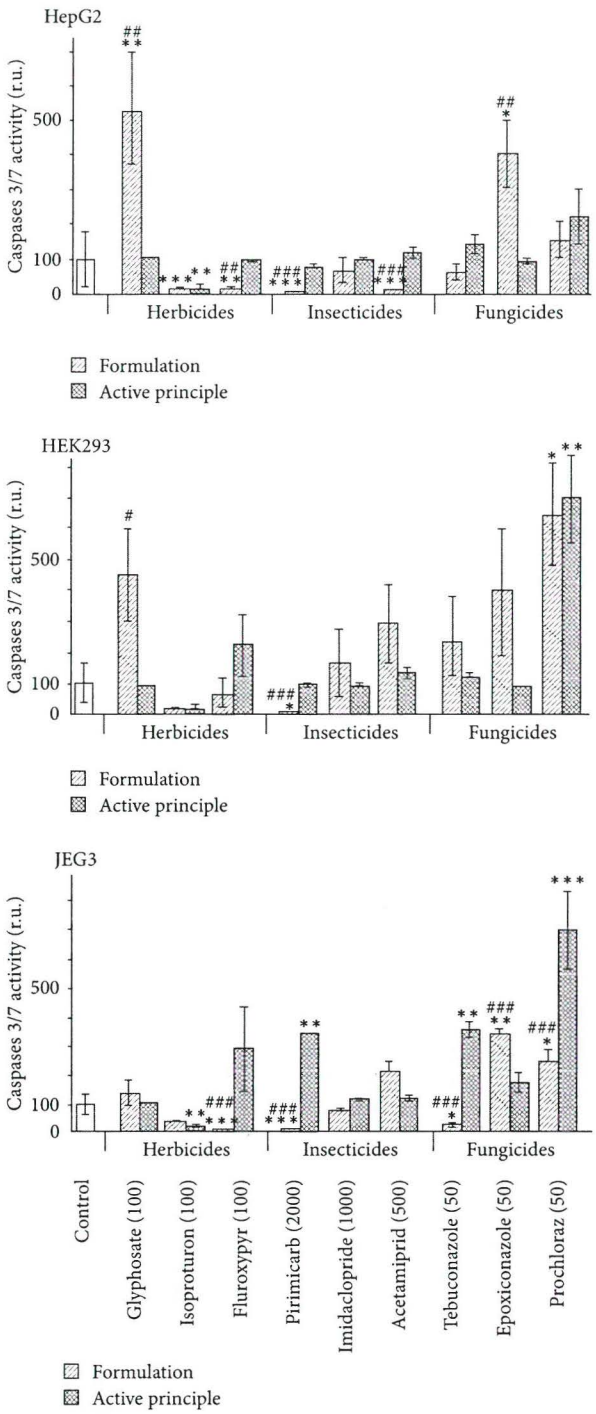

FIGURE 5: Differential apoptotic effects between formulations and their APs. The three described human cell lines were used in the culture conditions of Figure 1. We have chosen the doses at the first differential effects measured by MTT assay. SEMs are shown in all instances ($n = 9$). For the comparison of each AP or formulation to the control (white column), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in a nonparametric Mann-Whitney test. # symbol is used similarly for comparisons between APs and their formulations.

in toxicological evaluations of glyphosate-based herbicides. However, Roundup was found in this experiment to be 125 times more toxic than glyphosate. Moreover, despite its reputation, Roundup was by far the most toxic among the herbicides and insecticides tested. This inconsistency between scientific fact and industrial claim may be attributed to huge economic interests, which have been found to falsify health risk assessments and delay health policy decisions [41].

In conclusion, our results challenge the relevance of the ADI, because it is calculated today from the toxicity of the AP alone in vivo. An "adjuvant factor" of at least a reduction by 100 can be applied to the present calculation of the ADI if this is confirmed by other studies in vivo. As an example, the present ADI for glyphosate is 0.3 ppm; for glyphosate-based herbicides it would be 3 ppb or less. However, this will never replace the direct study of the commercial formulation with its adjuvants in regulatory tests. Anyway, an exposure to a single formulated pesticide must be considered as coexposure to an active principle and the adjuvants. In addition, the study of combinatorial effects of several APs together may be very secondary if the toxicity of the combinations of each AP with its adjuvants is neglected or unknown. Even if all these factors were known and taken into account in the regulatory process, this would not exclude an endocrine-disrupting effect below the toxicity threshold. The chronic tests of pesticides may not reflect relevant environmental exposures if only one ingredient is tested alone.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] R. Mesnage, B. Bernay, and G. E. Seralini, "Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity," *Toxicology*, vol. 313, no. 2-3, pp. 122–128, 2013.

[2] G. E. Seralini, R. Mesnage, N. Defarge et al., "Answers to critics: why there is a long term toxicity due to NK603 Roundup-tolerant genetically modified maize and to a Roundup herbicide," *Food and Chemical Toxicology*, vol. 53, pp. 461–468, 2013.

[3] C. Gasnier, C. Dumont, N. Benachour, E. Clair, M. Chagnon, and G. Séralini, "Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines," *Toxicology*, vol. 262, no. 3, pp. 184–191, 2009.

[4] M. Peluso, A. Munnia, C. Bolognesi, and S. Parodi, "32P-post-labeling detection of DNA adducts in mice treated with the herbicide Roundup," *Environmental and Molecular Mutagenesis*, vol. 31, no. 1, pp. 55–59, 1998.

[5] L. P. Walsh, C. McCormick, C. Martin, and D. M. Stocco, "Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression," *Environmental Health Perspectives*, vol. 108, no. 8, pp. 769–776, 2000.

[6] S. M. Bradberry, A. T. Proudfoot, and J. A. Vale, "Glyphosate poisoning," *Toxicological Reviews*, vol. 23, no. 3, pp. 159–167, 2004.

[7] A. Adam, A. Marzuki, H. A. Rahman, and M. A. Aziz, "The oral and intratracheal toxicities of ROUNDUP and its components to rats," *Veterinary and Human Toxicology*, vol. 39, no. 3, pp. 147–151, 1997.

[8] European Commission, "The use of plant protection products in the European Union," 2007, http://epp.eurostat.ec.europa.eu/.

[9] US EPA, "Pesticide Industry Sales and Usage," 2012, http://www.epa.gov/opp00001/pestsales/.

[10] A. Székács and B. Darvas, "Forty years with glyphosate," in *Herbicides-Properties, Synthesis and Control of Weeds*, M. N. A. E.-G. Hasaneen, Ed., InTech, Rijeka, Croatia, 2012.

[11] Commissariat Général au Développement Durable, *Les Pesticides Dans Les Milieux Aquatiques*, Études et Documents, Paris, France, 2010.

[12] P. R. Durkin, "Fluroxypyr human health and ecological risk assessment report," SERA TR-052-13-03a, USDA Forest service, Washington, DC, USA, 2009.

[13] J. Vera Candioti, G. S. Natale, S. Soloneski, A. E. Ronco, and M. L. Larramendy, "Sublethal and lethal effects on Rhinella Arenarum (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida®," *Chemosphere*, vol. 78, no. 3, pp. 249–255, 2010.

[14] M. L. Ambrose, *Characterization of the Insecticidal Properties of Acetamiprid Under Field and Laboratory Conditions*, Faculty of North Carolina State University, Raleigh, NC, US, 2003.

[15] L. Dicks, "Bees, lies and evidence-based policy," *Nature*, vol. 494, no. 7437, p. 283, 2013.

[16] Y. Aliouane, A. K. El Hassani, V. Gary, C. Armengaud, M. Lambin, and M. Gauthier, "Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior," *Environmental Toxicology and Chemistry*, vol. 28, no. 1, pp. 113–122, 2009.

[17] Agropages, "Triazole Fungicides Insight-Market overview," 2013, http://www.agropages.com/BuyersGuide/category/Triazole-Fungicides-Insight.html#smmn1.

[18] R. J. Letcher, I. Van Holsteijn, H. Drenth et al., "Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells," *Toxicology and Applied Pharmacology*, vol. 160, no. 1, pp. 10–20, 1999.

[19] C. Urani, M. Doldi, S. Crippa, and M. Camatini, "Human-derived cell lines to study xenobiotic metabolism," *Chemosphere*, vol. 37, no. 14-15, pp. 2785–2795, 1998.

[20] G. G. Kuiper, J. G. Lemmen, B. Carlsson et al., "Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β," *Endocrinology*, vol. 139, no. 10, pp. 4252–4263, 1998.

[21] J. Krijt, I. Van Holsteijn, I. Hassing, M. Vokurka, and B. J. Blaauboer, "Effect of diphenyl ether herbicides and oxadiazon on porphyrin biosynthesis in mouse liver, rat primary hepatocyte culture and HepG2 cells," *Archives of Toxicology*, vol. 67, no. 4, pp. 255–261, 1993.

[22] I. Nakagawa, M. Suzuki, N. Imura, and A. Naganuma, "Enhancement of paraquat toxicity by glutathione depletion in mice in vivo and in vitro," *Journal of Toxicological Sciences*, vol. 20, no. 5, pp. 557–564, 1995.

[23] S. Richard, S. Moslemi, H. Sipahutar, N. Benachour, and G. Seralini, "Differential effects of glyphosate and roundup on human placental cells and aromatase," *Environmental Health Perspectives*, vol. 113, no. 6, pp. 716–720, 2005.

[24] B. L'Azou, P. Fernandez, R. Bareille et al., "In vitro endothelial cell susceptibility to xenobiotics: comparison of three cell types," *Cell Biology and Toxicology*, vol. 21, no. 2, pp. 127–137, 2005.

[25] N. Benachour and G. Séralini, "Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells," *Chemical Research in Toxicology*, vol. 22, no. 1, pp. 97–105, 2009.

[26] H. C. Korting, S. Schindler, A. Hartinger, M. Kerscher, T. Angerpointner, and H. I. Maibach, "MTT-assay and neutral red release (NRR)-assay: relative role in the prediction of the irritancy potential of surfactants," *Life Sciences*, vol. 55, no. 7, pp. 533–540, 1994.

[27] J. J. Liu, W. Wang, D. T. Dicker, and W. S. El-Deiry, "Bioluminescent imaging of TRAIL-induced apoptosis through detection of caspase activation following cleavage of DEVD-aminoluciferin," *Cancer Biology & Therapy*, vol. 4, no. 8, pp. 885–892, 2005.

[28] S. P. Crouch, R. Kozlowski, K. J. Slater, and J. Fletcher, "The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity," *Journal of Immunological Methods*, vol. 160, no. 1, pp. 81–88, 1993.

[29] N. Benachour, H. Sipahutar, S. Moslemi, C. Gasnier, C. Travert, and G. E. Séralini, "Time- and dose-dependent effects of roundup on human embryonic and placental cells," *Archives of Environmental Contamination and Toxicology*, vol. 53, no. 1, pp. 126–133, 2007.

[30] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[31] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "NIH Image to ImageJ: 25 years of image analysis," *Nature Methods*, vol. 9, no. 7, pp. 671–675, 2012.

[32] M. Marutani and V. Edirveerasingam, "Influence of irrigation methods and an adjuvant on the persistence of carbaryl on pakchoi," *Journal of Environmental Quality*, vol. 35, no. 6, pp. 1994–1998, 2006.

[33] R. H. McKee, Z. A. Wong, S. Schmitt et al., "The reproductive and developmental toxicity of high flash aromatic naphtha," *Toxicology and Industrial Health*, vol. 6, no. 3-4, pp. 441–460, 1990.

[34] J. M. Langman, "Xylene: its toxicity, measurement of exposure levels, absorption, metabolism and clearance," *Pathology*, vol. 26, no. 3, pp. 301–309, 1994.

[35] A. M. Saillenfait, F. Gallissot, I. Langonné, and J. P. Sabaté, "Developmental toxicity of N-methyl-2-pyrrolidone administered orally to rats," *Food and Chemical Toxicology*, vol. 40, no. 11, pp. 1705–1712, 2002.

[36] US Environmental Protection Agency (EPA), "Hazard Characterization Document-N, N-Dimethylalkanamides Category," 2011, http://www.epa.gov/chemrtk/hpvis/hazchar/Category_N,N-Dimethylalkanamides_September_2011 .pdf.

[37] US Environmental Protection Agency (EPA), *Reregistration Eligibility Decision (RED) For Benzisothiazoline-3-One*, US Environmental Protection Agency (EPA), Boston, Mass, USA, 2005.

[38] L. Gawade, S. S. Dadarkar, R. Husain, and M. Gatne, "A detailed study of developmental immunotoxicity of imidacloprid in Wistar rats," *Food and Chemical Toxicology*, vol. 51, pp. 61–70, 2013.

[39] G. M. Williams, R. Kroes, and I. C. Munro, "Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans," *Regulatory Toxicology and Pharmacology*, vol. 31, no. 2, part 1, pp. 117–165, 2000.

[40] A. L. Williams, R. E. Watson, and J. M. Desesso, "Developmental and reproductive outcomes in humans and animals after glyphosate exposure: a critical analysis," *Journal of Toxicology and Environmental Health B*, vol. 15, no. 1, pp. 39–96, 2012.

[41] European Environment Agency, "Late lessons from early warnings: science, precaution, innovation," EEA Report, European Environment Agency, Copenhagen, Denmark, 2013.

An Exploratory Analysis of the Effect of Pesticide Exposure on the Risk of Spontaneous Abortion in an Ontario Farm Population

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The toxicity of pesticides on human reproduction is largely unknown—particularly how mixtures of pesticide products might affect fetal toxicity. The Ontario Farm Family Health Study collected data by questionnaire on the identity and timing of pesticide use on the farm, lifestyle factors, and a complete reproductive history from the farm operator and eligible couples living on the farm. A total of 2,110 women provided information on 3,936 pregnancies, including 395 spontaneous abortions. To explore critical windows of exposure and target sites for toxicity, we examined exposures separately for preconception (3 months before and up to month of conception) and postconception (first trimester) windows and for early (< 12 weeks) and late (12–19 weeks) spontaneous abortions. We observed moderate increases in risk of early abortions for preconception exposures to phenoxy acetic acid herbicides [odds ratio (OR) = 1.5; 95% confidence interval (CI), 1.1–2.1], triazines (OR = 1.4; 95% CI, 1.0–2.0), and any herbicide (OR = 1.4; 95% CI, 1.1–1.9). For late abortions, preconception exposure to glyphosate (OR = 1.7; 95% CI, 1.0–2.9), thiocarbamates (OR = 1.8; 95% CI, 1.1–3.0), and the miscellaneous class of pesticides (OR = 1.5; 95% CI, 1.0–2.4) was associated with elevated risks. Postconception exposures were generally associated with late spontaneous abortions. Older maternal age (> 34 years of age) was the strongest risk factor for spontaneous abortions, and we observed several interactions between pesticides in the older age group using Classification and Regression Tree analysis. This study shows that timing of exposure and restricting analyses to more homogeneous endpoints are important in characterizing the reproductive toxicity of pesticides. ***Key words:*** **atrazine, carbaryl, developmental toxicity, epidemiologic methods, glyphosate, herbicides, pesticides, phenoxy acetic acid herbicides, spontaneous abortion, thiocarbamates, triazine, windows of vulnerability.** *Environ Health Perspect* 109:851–857 (2001). [Online 14 August 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p851-857arbuckle/abstract.html

Farm residents may be exposed to several types of pesticides from various chemical families (e.g., phenoxy acetic acids, triazines, carbamates, and organophosphates) during the course of a growing season. Several studies have reported positive associations between occupational pesticide exposure and fetal death (spontaneous abortion or stillbirth) (*1–3*). However, little is known about the human reproductive toxicity of specific pesticide active ingredients and even less about mixtures of pesticides and how they may interact with other risk factors.

In addition to the nature of the chemical and its target, the consequences of exposure to chemical agents depend on the timing of exposure relative to critical windows in development of the fetus or reproductive system (*4,5*). In a recent article (*6*), we noted that the risk of spontaneous abortion in farm families varied depending on when exposure to phenoxy herbicides occurred and on whether the abortion occurred earlier (< 12 weeks) or later (12–19 weeks) in the pregnancy. Previous analyses had also discussed the role of male pesticide exposure on pregnancy outcomes (*7*) and time to pregnancy (*8*). In this analysis we used the data from our study of farm families to explore further the critical windows of exposure, the target sites and interaction among the pesticides, and other risk factors for spontaneous abortion.

Subjects and Methods

The Ontario Farm Family Health Study collected data retrospectively by questionnaire from farm operators and eligible couples living on the selected farms, as described in detail elsewhere (*6,9*). To be eligible, the couple had to be living year round on the study farm and the wife had to be 44 years of age or younger (to reduce the length of recall of reproductive events). At least one member of the couple had to be working on the farm. Three questionnaires were designed to collect relevant information from the farm operator, husband, and wife on demographic and lifestyle information; pesticides currently and historically used on the farm and around the home; medical history; and a complete reproductive history.

The women in the study were asked to recall all their pregnancies, starting with their first. For spontaneous abortions, the woman was asked how many weeks pregnant she was (based on the last menstrual period) at the time of the abortion. We calculated the estimated calendar month of conception by subtracting the gestational age at abortion or delivery from the delivery date. The outcome of interest in this analysis was self-reported spontaneous abortion of less than 20 weeks' gestation. We examined subgroups of spontaneous abortions of less than 12 weeks' and 12–19 weeks' gestation to provide an indirect estimate of risk by likely frequency of chromosomal anomaly, a more common cause in early abortions (*10*). Pregnancies occurring when the woman was not living on the study farm and thus had unknown exposure status were excluded, as were pregnancies for which the study husband was not likely the father.

We pooled pesticide exposure information from the farm operator (the person responsible for the day-to-day operations of the farm, if different from the husband or wife), husband, and wife to construct a history of monthly agricultural and residential pesticide use. For each pesticide reported, we identified the active ingredients and uses using a database of registered pesticide products in Canada. Where possible, we categorized the active ingredients into chemical families. We divided all pesticides reported into four major classes of use: herbicides, insecticides, fungicides, and miscellaneous others (including those that could not be classified). We identified the active ingredients and chemical families that were most frequently used on the farms in the study, as well as those most likely to have adverse reproductive effects according to the literature. This categorization produced 17 pesticide unit variables that we examined in this study (Table 1).

Because only couples living on the farm were eligible for the study, the exposure assessment in this analysis was intended to capture potential occupational (direct) and residential (indirect) exposures. Because

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indirect exposures were possible, we could not completely separate the exposure statuses of the men and women. Most pesticide applications were done by the husband, with only 20% of the wives reporting handling of farm pesticides. No other information was available to validate the exposure assessments; however, we used both open-ended and checklist questions to obtain as complete a recall as possible.

We merged reproductive and pesticide exposure history data to create pesticide unit variables for months preceding and during each pregnancy. Exposure to pesticides was analyzed for two windows: preconception, the 4-month period from 3 months before conception to the calendar month of conception (consistent with potential sperm-mediated effects); and postconception, the 3-month period from the first calendar month after conception to the end of the first trimester (consistent with a fetotoxic effect). Exposures that occurred after a pregnancy loss but within the period of interest (i.e., first trimester) were not considered in assessing exposure status. We also created pregnancy-specific variables for all other time-related factors (parental age, smoking, farm activities, and alcohol and caffeine intake).

Statistical Analysis

We calculated crude odds ratios (ORs) using logistic regression for each combination of pesticide unit, exposure window, and gestational age at abortion category. Because no strong confounders were evident in previous analyses of these data (*6*) and our sample size was limited, we did not estimate adjusted risks. Nonexposed pregnancies were those not exposed during the time window to the pesticide unit of interest.

To assess the importance of the timing of exposure to the risk of spontaneous abortion, we compared preconception exposures to postconception in a combined model where preconception exposures were coded 1 and postconception exposures were the referent. Pregnancies exposed to a pesticide unit in both windows were excluded from this analysis. Similarly, we used an indicator to distinguish early (< 12 weeks' gestation) and late (12–19 weeks' gestation) fetal age at abortion to identify the major target site for pesticide toxicity (embryo or fetus). In this latter model, which analyzed only spontaneous abortions, we used the 12–19 weeks' gestational age abortions as the referent group.

To explore statistical interactions between the various pesticide units and other risk factors for spontaneous abortion, we used the Classification and Regression Tree (CART) method. This method has been discussed in detail by Breiman and colleagues (*11*). The CART method has been applied in other disciplines, for example in diagnosing chest pain (*12*) and recently in epidemiologic studies (*13,14*).

CART is a nonparametric method used to construct a classification rule for predicting what class of an object or case is based on the values of its predictor variables. A tree is constructed by recursively partitioning a data set into increasingly homogeneous (measured by the distribution of the outcome variable) descendant subsets (*11*). Partitioning is conducted using a single covariate at a time and is represented by a node (branch) in the tree. The top node of the tree is called the root node. Those nodes that are not split are called terminal nodes or leaves.

Our search for interaction effects using CART involved all 17 pesticides variables analyzed separately for each level (use class, chemical family, and active ingredient), as well as 21 possible risk factors for spontaneous abortion (e.g., maternal and paternal age, education, smoking status, alcohol and caffeine consumption, and family income).

Our small sample size prevented subgroup analyses of early and late spontaneous abortions. CART analysis was conducted using a commercially developed software, AnswerTree 2.0 (*15*). The Gini criterion was applied in the selection of best splits. ORs and 95% confidence intervals (CI) were calculated for each node using SAS (*16*).

Table 1. The 17 pesticide unit variables created in the Ontario Farm Family Health Study.

Pesticide use class	Chemical family	Active ingredient
Herbicide		Dicamba
		Glyphosate
	Phenoxy acetic acid (Phenoxy herbicides)	4-[2,4-dichlorophenoxy] butyric acid (2,4-DB)
		2,4-dichlorophenoxyacetic acid (2,4-D)
		[4-chloro-2-methylphenoxy] acetic acid (MCPA)
	Triazine	Atrazine
		Cyanazine
	Organophosphate	
	Thiocarbamate	
Insecticide		Carbaryl
	Organophosphate	
Fungicide		Captan
	Thiocarbamate	
	Triazine	
Miscellaneous		

Results

Approximately 2,000 farm couples participated in the study, contributing 3,936 pregnancies for analysis including 395 spontaneous abortions. All but five of the abortions were reported by the women as medically confirmed. Mean gestational age was 10 weeks, with 57% of the spontaneous abortions occurring before 12 weeks' gestation. The women participating in the study were involved to varying extents in working on the farm. Forty-eight percent assisted with the harvesting of crops, 21% milked cows, 20% helped to prepare the land for planting, and 3% applied crop herbicides. The wives were generally better educated than their husbands, with almost 40% having a college or university degree compared to 28% for the men. More farm men (22%) than women (16%) were current smokers. More than 70% of the farm women drank less than one alcoholic beverage per week, whereas about 43% of the men drank at least once per week.

Critical Exposure Window

Although many of the results shown in Table 2 are not statistically significant, preconception exposure to glyphosate, triazines, thiocarbamates, herbicides, fungicides, and miscellaneous pesticides moderately increased the risk for all spontaneous abortions (< 20 weeks). When the analysis was restricted to early abortions (< 12 weeks), increased risks were observed for preconception exposure to phenoxy acetic acid herbicides (OR = 1.5; 95% CI, 1.1–2.1) and two of its constituents, 2,4-dichlorophenoxyacetic acid (2,4-D) (OR = 1.3; 95% CI, 0.9–2.0) and 2,4-DB (OR = 1.4; 95% CI, 0.7–2.8), in addition to the triazine chemical family and herbicide class of pesticides. For late spontaneous abortions (12–19 weeks), preconception exposure to thiocarbamates (OR = 1.8; 95% CI, 1.1–3.0), glyphosate (OR = 1.7; 95% CI, 1.0–2.9), fungicides (OR = 1.4; 95% CI, 0.9–2.1), and the miscellaneous class of pesticides (OR = 1.5; 95% CI, 1.0–2.4) were associated with elevated risks.

Risk estimates for the postconception exposure window are listed in Table 3. The risks associated with the miscellaneous class of pesticides were elevated for both early and late spontaneous abortions. Other elevations in risk were observed only in the late abortions after exposure to 2,4-D (OR = 1.6; 95% CI, 0.9–2.7), dicamba (OR = 1.6; 95% CI, 0.8–3.2), glyphosate (OR = 1.4; 95% CI, 0.8–2.5), and the phenoxy acetic acid herbicides (OR = 1.3; 95% CI, 0.8–2.0).

For most pesticides examined, preconception exposure contributed more to the risk of a spontaneous abortion than exposures during the first trimester. This was especially true for early abortions, as measured by the elevated odds ratios observed when models were constructed with exposure window as the outcome (Table 4). Analyses that incorporated gestational age at abortion as the outcome variable generally produced higher risk estimates for early spontaneous abortions from preconception exposure (Table 5). Except for cyanazine, carbaryl, and organophosphates, postconception exposures had more effect on the risk of late abortions, as measured by odds ratios less than one.

Interaction among Risk Factors

Overall, in the tree-based analysis, maternal age was the strongest risk factor observed for spontaneous abortions of less than 20 weeks' gestation. Maternal age partitioned the study population with a cut-off of 35 years of age. A pregnant woman age 35 or older was 2.6 times more likely to have a spontaneous abortion than a younger woman (95% CI, 1.7–3.9).

Among older women, preconception exposure to carbaryl and 2,4-D determined further refinement of these subgroups (Figure 1). Women age 35 or older who were exposed to carbaryl had nearly a 4-fold increase in risk compared to women of the same age who were not exposed. Pregnancies of women less than 35 years of age (node 2) were not at increased risk of a spontaneous abortion if exposed to any of the active ingredients during the preconception window. Node 2 is called a terminal node (or leaf) because further splitting could not generate an odds ratio different from one. Node 5 is further split into nodes 6 and 7. Based on a comparison of nodes 6 and 7, a pregnant woman 35 years or older exposed to both carbaryl and 2,4-D was 27 times more likely to have a spontaneous abortion than a woman in the same age range who was exposed to carbaryl only.

When the analysis was conducted at the chemical family level, we detected interaction effects between maternal age and preconception exposure to several pesticide families (Figure 2). The results suggested that a pregnant women age 35 or older who is exposed to triazines during the preconception window had nearly three times the risk (OR = 2.7; 95% CI, 1.1–6.9) of a spontaneous abortion. Furthermore, from nodes 6 and 7, we observed that preconception exposure to phenoxy acetic acid herbicides in the older group of women more than doubled the risk (OR = 2.3; 95% CI, 0.6–8.6). At nodes 8 and 9, we observed a three-way interaction effect among maternal age, triazines, and thiocarbamates, indicating that a pregnant woman 35 years or older who was exposed to both triazines and thiocarbamates before conception had a nearly 8-fold increase in risk over those exposed to triazines only. No such interaction was observed for younger women.

We also observed interactions between pesticide use classes (data not shown). Exposure to both fungicides and herbicides before conception doubled the risk relative to that for a woman who was exposed only to fungicides (OR = 2.0; 95% CI, 1.1–3.5). Among the older group of pregnant women, exposure to fungicides doubled the risk of having a spontaneous abortion compared to those not exposed (OR = 2.4; 95% CI, 1.0–5.9). No increased risk was observed among the younger women.

Interactions with maternal age were also found among postconception exposures to pesticides. Among older women exposed to glyphosate, the risk was three times that for women of the same age who were not exposed to this active ingredient (OR = 3.2; 95% CI, 0.8–23.0). Pregnant women age 35 or older exposed during the first trimester to thiocarbamates were at increased risk of spontaneous abortion (OR = 2.4; 95% CI, 0.5–10.5). Younger women exposed to the same chemical family were not at increased risk of an abortion. Pregnant women 35 or older exposed during pregnancy to the miscellaneous class of pesticides were at increased risk (OR = 2.5; 95% CI, 0.9–6.7).

Table 2. Spontaneous abortion risk and preconception exposure to various pesticides.

	All gestational ages	< 12 weeks		12–19 weeks	
Pesticide unit	Crude OR (95% CI)	No. of exposed cases[a]	Crude OR (95% CI)	No. of exposed cases[a]	Crude OR (95% CI)
Pesticide active ingredient					
Atrazine	1.2 (0.9–1.7)	24	1.3 (0.8–2.0)	16	1.1 (0.7–1.9)
Captan	1.0 (0.5–1.8)	6	1.0 (0.4–2.1)	5	1.0 (0.4–2.6)
Carbaryl	1.2 (0.9–1.7)	24	1.2 (0.8–1.9)	17	1.2 (0.7–2.0)
Cyanazine	0.7 (0.3–1.7)	4	0.9 (0.3–2.4)	2	0.6 (0.1–2.3)
2,4-D	1.2 (0.8–1.6)	26	1.3 (0.9–2.0)	13	0.9 (0.5–1.6)
2,4-DB	0.8 (0.4–1.5)	10	1.4 (0.7–2.8)	0	0.1 (0.0–1.4)
Dicamba	1.0 (0.7–1.7)	11	1.0 (0.5–1.8)	9	1.1 (0.6–2.2)
Glyphosate	1.4 (1.0–2.1)	16	1.1 (0.7–1.9)	17	1.7 (1.0–2.9)
MCPA	0.8 (0.5–1.3)	17	1.1 (0.6–1.8)	7	0.6 (0.3–1.2)
Chemical families					
Phenoxy acetic acid	1.2 (0.9–1.5)	48	1.5 (1.1–2.1)	21	0.8 (0.5–1.9)
Triazine	1.3 (1.0–1.8)	35	1.4 (1.0–2.0)	22	1.1 (0.7–1.8)
Organophosphate	1.0 (0.7–1.4)	24	1.0 (0.6–1.6)	18	1.0 (0.6–1.7)
Thiocarbamate	1.5 (1.0–2.1)	16	1.1 (0.7–1.9)	18	1.8 (1.1–3.0)
Use classes					
Herbicide	1.3 (1.0–1.6)	78	1.4 (1.1–1.9)	51	1.1 (0.8–1.6)
Insecticide	1.1 (0.9–1.4)	68	1.2 (0.9–1.5)	49	1.1 (0.8–1.5)
Fungicide	1.4 (1.1–1.8)	36	1.3 (0.9–1.9)	28	1.4 (0.9–2.1)
Miscellaneous	1.5 (1.1–2.0)	25	1.3 (0.8–2.1)	21	1.5 (1.0–2.4)

[a]The total number of cases of spontaneous abortion is 395, with 226 and 169 early and late abortions, respectively.

Table 3. Spontaneous abortion risk and postconception exposure to various pesticides.

	All gestational ages	< 12 weeks		12–19 weeks	
Pesticide unit	Crude OR (95% CI)	No. of exposed cases[a]	Crude OR (95% CI)	No. of exposed cases[a]	Crude OR (95% CI)
Pesticide active ingredient					
Atrazine	0.8 (0.5–1.2)	10	0.7 (0.3–1.5)	8	0.8 (0.4–1.6)
Captan	0.6 (0.3–1.4)	2	0.3 (0.1–1.4)	4	0.9 (0.3–2.5)
Carbaryl	0.8 (0.5–1.2)	14	0.9 (0.5–1.6)	7	0.6 (0.3–1.3)
Cyanazine	0.1 (0.0–0.9)	1	0.2 (0.0–1.4)	0	0.1 (0.0–2.4)
2,4-D	1.0 (0.7–1.6)	9	0.6 (0.3–1.2)	16	1.6 (0.9–2.7)
2,4-DB	0.4 (0.2–1.1)	1	0.2 (0.0–1.2)	3	0.7 (0.2–2.3)
Dicamba	1.1 (0.7–1.9)	6	0.8 (0.3–1.7)	9	1.6 (0.8–3.2)
Glyphosate	1.1 (0.7–1.7)	10	0.8 (0.4–1.6)	12	1.4 (0.8–2.5)
MCPA	0.8 (0.5–1.3)	8	0.7 (0.3–1.4)	8	0.9 (0.4–1.8)
Chemical families					
Phenoxy acetic acid	0.9 (0.6–1.2)	16	0.6 (0.4–1.0)	23	1.3 (0.8–2.0)
Triazine	0.7 (0.4–1.1)	12	0.6 (0.4–1.1)	11	0.8 (0.4–1.5)
Organophosphate	0.6 (0.4–1.0)	10	0.5 (0.3–1.0)	12	0.9 (0.5–1.5)
Thiocarbamate	0.8 (0.5–1.3)	7	0.6 (0.3–1.3)	9	1.1 (0.5–2.2)
Use classes					
Herbicide	0.8 (0.7–1.1)	37	0.7 (0.5–1.0)	38	1.1 (0.7–1.5)
Insecticide	0.8 (0.6–1.1)	40	0.7 (0.5–1.1)	37	1.0 (0.8–1.4)
Fungicide	0.8 (0.5–1.1)	16	0.6 (0.4–1.0)	18	1.0 (0.6–1.6)
Miscellaneous	1.7 (1.2–2.3)	25	1.4 (0.9–2.2)	24	1.9 (1.2–3.0)

[a]The total number of cases of spontaneous abortion is 395, with 226 and 169 early and late abortions, respectively.

The odds ratio for younger women exposed to the same group of chemicals was 1.5 (95% CI, 1.1–2.2). Furthermore, the risk for pregnant women 35 or older exposed to both miscellaneous pesticides and fungicides was 4.3 (95% CI, 0.3–57.6).

Discussion

Our results suggest that the critical window of exposure for spontaneous abortions of less than 20 completed weeks of gestation is during the 4-month period from 3 months before conception up to and including the calendar month of conception. Preconception exposure to the pesticide active ingredients glyphosate, atrazine, carbaryl, and 2,4-D was associated with a 20–40% relative increase in risk; whereas postconception exposures to any of the pesticide units tested (except the miscellaneous class of pesticides) was not associated with an increased risk. Pesticides belonging to the triazine, thiocarbamate, or phenoxy acetic acid chemical families were also associated with moderately increased risks.

Analysis of early (< 12 weeks) and late (12–19 weeks) spontaneous abortions revealed differences between the timing of exposure and the target, represented by the gestational age at abortion. Preconception exposure to the triazine (atrazine) and phenoxy herbicides (2,4-D and 2,4-DB) was associated with increased risks of early but not late spontaneous abortion. The herbicide glyphosate was associated with increased risks of late abortion, regardless of when exposure occurred. Generally, pregnancies exposed to pesticides before conception resulted in early abortions, suggesting a paternally mediated mechanism. There was some indication, measured by our comparison modeling, that postconception exposures were more likely associated with late abortions.

This finding has important implications for our understanding of the mechanism by which chemical exposures may cause spontaneous abortions. Previous studies have already suggested the existence of etiologic differences between early and late spontaneous abortions (*10,17*). Most early abortions have gross chromosomal anomalies (*18*). Our findings of an association between preconception exposure and an early abortion may imply that for some pesticides, preconception exposures lead to gross chromosomal anomalies. On the other hand, our finding of an association between late abortions and postconception exposure may suggest that postconception exposure to specific pesticides tends to damage the fetus or fetus–placenta complex rather than cause chromosomal anomalies.

We also found strong evidence of interaction between maternal age and pesticide exposure on the risk of spontaneous abortion in both exposure windows. Most of the increased risks associated with pesticide exposure were observed in women age 35 or older. Similar to the findings of other studies (*19,20*), we observed that advanced maternal age was associated with an increased risk of spontaneous abortion (crude OR = 2.6; 95% CI, 1.7–3.9). Trisomic oocytes and a less efficient uterus have been identified as independent risks for older women (*21*). Maternal age may also be a surrogate measure for cumulative exposure to various pesticides, other unknown factors, or accumulated toxicity for either parent, because it is often highly correlated with paternal age.

Although several epidemiologic studies of the reproductive toxicity of pesticides have been conducted suggesting increased risks of fetal deaths, few have focused on specific pesticide products or chemical families (*22*). The phenoxy herbicides have been one of the most commonly studied groups of pesticides. Genetic *in vitro* toxicity testing on the phenoxy herbicide 2,4-D has reportedly been negative (*23*). Paternally mediated reproductive toxicity of a picloram and 2,4-D combination herbicide has been suggested in mice (*24*). Human studies have shown that this pesticide may damage sperm (*25*), increase the risk of spontaneous abortion in wives of older farmers (ages 31–35) (*26*), and be measured in seminal fluid of applicators (*27*).

The triazine pesticide atrazine has caused chromosomal damage in Chinese hamster ovary cells (*28*) and been associated with elevated rates of intrauterine growth retardation in communities with contaminated drinking waters (*29*). However, there is conflicting evidence as to whether atrazine is mutagenic in cultured human cell lines (*30–33*). Atrazine has had adverse reproductive effects in rats, including fetal losses (*34*). Cyanazine has shown some teratogenic effects in rats (*35*).

The genotoxicity of glyphosate has been positive in *in vitro* cultures of bovine (*36*) and human lymphocytes (*32*) and weakly mutagenic in a *Salmonella* assay (*37*). Carbaryl, a carbamate pesticide, has been associated with increased risks of childhood brain cancer (*38*)

Table 4. Comparison analysis of effects of pre- versus postconception exposure to pesticides on spontaneous abortion.[a]

Pesticide unit	All gestational ages Crude OR[b] (95% CI)	< 12 weeks Preconception exposed cases	< 12 weeks Postconception exposed cases	< 12 weeks Crude OR (95% CI)	12–19 weeks Preconception exposed cases	12–19 weeks Postconception exposed cases	12–19 weeks Crude OR (95% CI)
Pesticide active ingredient							
Atrazine	1.6 (0.9–3.0)	23	9	1.7 (0.8–3.8)	15	7	1.4 (0.6–3.5)
Captan	2.2 (0.6–8.5)	5	1	4.0 (0.5–35.4)	3	2	1.1 (0.2–7.1)
Carbaryl	2.0 (1.0–4.0)	17	7	1.7 (0.7–4.4)	15	5	2.2 (0.8–6.1)
Cyanazine	5.6 (0.7–47.5)	4	1	3.6 (0.4–33.1)	2	0	4.4 (0.2–94.3)
2,4-D	1.1 (0.6–2.0)	22	5	2.9 (1.1–8.0)	11	14	0.5 (0.2–1.1)
2,4-DB	1.8 (0.6–6.2)	10	1	7.8 (1.0–62.3)	0	3	0.1 (0.0–2.0)
Dicamba	1.0 (0.4–1.9)	9	4	1.4 (0.4–4.7)	8	8	0.6 (0.2–1.6)
Glyphosate	1.6 (0.7–3.4)	12	6	1.7 (0.6–4.2)	10	5	1.5 (0.5–4.6)
MCPA	1.1 (0.6–2.3)	14	5	2.0 (0.7–5.7)	7	8	0.6 (0.2–1.7)
Chemical families							
Phenoxy acetic acid	1.3 (0.8–2.1)	41	9	3.1 (1.4–6.4)	19	21	0.6 (0.3–1.1)
Triazine	1.9 (1.0–3.2)	34	11	2.1 (1.0–4.4)	20	9	1.4 (0.7–3.2)
Organophosphate	2.2 (1.0–4.8)	17	3	3.8 (1.1–13.4)	12	6	1.3 (0.4–3.6)
Thiocarbamate	2.5 (1.1–5.8)	13	4	2.0 (0.8–5.0)	13	4	2.4 (0.8–7.6)
Use classes							
Herbicide	1.6 (1.1–2.4)	59	18	2.3 (1.3–3.9)	36	23	1.1 (0.7–1.8)
Insecticide	1.7 (1.1–2.9)	38	10	2.6 (1.3–5.2)	28	16	1.2 (0.6–2.2)
Fungicide	2.8 (1.4–5.4)	25	5	3.9 (1.4–10.3)	17	7	1.8 (0.7–4.4)
Miscellaneous	0.6 (0.3–1.4)	5	5	0.8 (0.2–3.0)	5	8	0.5 (0.2–1.6)

Pregnancies with both pre- and postconception exposure have been excluded from the analysis in this table.
[a]Postconception exposure window used as referent group. [b]The odds ratios estimate the risk that exposures to pesticides resulting in a spontaneous abortion occurred in the preconception window, relative to the postconception window.

and reproductive and developmental effects in animals (*39*). Captan may be a potential clastogenic agent (*40*).

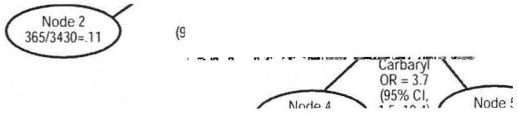
There is evidence that organophosphate pesticides have genotoxic effects in humans (*41*). Workers in Chinese pesticide factories exposed to organophosphate pesticides had moderately increased prevalences of sperm aneuploidy (*42*). Methamidophos, an organophosphate, may have the potential to affect male fertility and to produce transmissible adverse embryonic effects after an acute paternal germline exposure (*43*).

Although this study is one of the first to collect and analyze detailed information on the timing and types of pesticides used on farms and reproductive outcomes, several limitations suggest that our findings be interpreted with caution. Because dose information was not available, misclassification of exposure is likely. Many factors including the pesticide formulation, application conditions, handling practices, and interindividual differences in absorption, distribution, metabolism, and excretion of the products or metabolites will lead to variability in the degree of exposure. Because the farmers used many different pesticides during the study and our sample size was limited, findings may be unreliable, particularly for multiple pesticide interactions. Because pesticide products were reported primarily by the farm applicator or husband, differential recall of pesticide exposure by the mother is not likely to be a problem in this study; however, some nondifferential recall of pesticides and spontaneous abortions is likely. Because the analyses were designed to generate, not to test, hypotheses, and multiple comparisons were conducted, results should be interpreted with care and tested in other studies.

Also worthy of consideration is the fact that couples contributed multiple pregnancies to the analyses, and pregnancies from the same woman are not independent events. In previous analyses (*6, 7*), generalized estimating equation models were constructed to account for this nonindependence and were found to have a modest effect on the confidence interval with little consequence on the effect measure. We also did not control for history of prior spontaneous abortion because these losses might have been caused partly by pesticide exposure and resulted in biased risk estimates (*44*). However, poor outcomes in previous pregnancies might alter behavior in subsequent pregnancies; for example, the woman might be more careful to avoid exposures to perceived toxic agents after experiencing a spontaneous abortion. Because this study has no personal pesticide dose information for either parent, we cannot rule out this potentially modifying effect. All the exposure information pertained to certain pesticides that were reported by either the farm operator or couple (mostly by the farm operator) as being used on the farm during a particular calendar period. We did not have information on the specific dates that each pesticide was applied, nor did we expect that the farm operators would be able to report these dates accurately. Consequently, depending on when during the calendar month conception occurred, exposure during the estimated month of conception may have been incorrectly assigned to the preconception window. In an earlier article (*6*) we looked at variations in the time window of interest. The pattern of risks during the estimated

Table 5. Odds of early versus late spontaneous abortion after exposure to pesticides at different times.[a]

	Preconception exposure			Postconception exposure		
Pesticide unit	No. cases < 12 weeks	No. cases 12–19 weeks	Crude OR[b] (95% CI)	No. cases < 12 weeks	No. cases 12–19 weeks	Crude OR (95% CI)
Active ingredient						
Atrazine	24	16	1.1 (0.6–2.2)	10	8	0.9 (0.4–2.4)
Captan	6	5	0.9 (0.3–2.9)	2	4	0.4 (0.1–2.0)
Carbaryl	24	17	1.1 (0.6–2.1)	14	7	1.5 (0.6–3.8)
Cyanazine	4	2	1.5 (0.3–8.3)	1	0	2.2 (0.1–55.6)
2,4-D	26	13	1.6 (0.8–3.1)	9	16	0.4 (0.2–0.9)
2,4-DB	10	0	16.4 (0.9–282.0)	1	3	0.2 (0.0–2.4)
Dicamba	11	9	0.9 (0.4–2.2)	6	9	0.5 (0.2–1.4)
Glyphosate	16	17	0.7 (0.3–1.4)	10	12	0.6 (0.2–1.4)
MCPA	17	7	1.8 (0.8–4.6)	8	8	0.7 (0.3–2.0)
Chemical families						
Phenoxy acetic acid	48	21	1.9 (1.1–3.3)	16	23	0.5 (0.2–0.9)
Triazine	35	22	1.2 (0.7–2.2)	12	11	0.8 (0.3–1.9)
Organophosphate	24	18	1.0 (0.5–1.9)	10	12	1.6 (0.3–1.4)
Thiocarbamate	16	18	0.6 (0.3–1.3)	7	9	0.6 (0.2–1.6)
Use classes						
Herbicide	78	51	1.2 (0.8–1.9)	37	38	0.7 (0.4–1.1)
Insecticide	68	49	1.1 (0.7–1.6)	40	37	0.8 (0.5–1.3)
Fungicide	36	28	1.0 (0.6–1.6)	16	18	0.6 (0.3–1.3)



abortion risk (< 20 we
active ingredients and

[a]In the nodes, the numerators represent the number of cases, the den number of non-cases. [b]Left branch of node used as referent group.

calendar month of conception was included in the preconception window in the current analyses.

Our analyses did not consider the half-lives of the individual pesticides. Several of the herbicides, such as those in the phenoxy family, have relatively short half-lives, whereas others may have longer half-lives or persist in the environment. In addition, we examined only the active ingredients, not the so-called inert ingredients in pesticide products. Some of the inert ingredients may contribute to the potential toxicity of the pesticide product. Unfortunately, much of this information is not readily available.

The referent group in most of the analyses reported here (Tables 2 and 3) comprised pregnancies not exposed to the pesticide of interest during the window under consideration. In an earlier article (*6*) in which the referent group was pregnancies not exposed to any pesticides during the window, we reported a crude odds ratio for early abortions of 2.3 (95% CI, 1.0–5.6) for preconception exposure to phenoxy herbicides. Here we report an odds ratio of 1.5 (95% CI, 1.1–2.1), showing the attenuation in risk when a different referent group is used.

Exploring statistical interaction between pesticides and other risk factors is one of the contributions of this article to the literature. Previous studies have lacked sufficient detail on pesticide products to allow for such a comparison. The statistical techniques most commonly used to assess statistical interaction and to control for confounders are logistic regression and stratified analysis (*45*). However, these two methods are designed primarily for hypothesis or theory testing with few predictor variables. In an exploratory study of statistical interaction, both methods are extremely time consuming when the number of combinations of two-way or three-way interactions is large (*46*).

The CART method has several advantages over traditional methods in an exploratory study, especially with a large data set (*12,14,47*). It helps researchers identify important predictor variables and cut points for continuous variables. It can also detect various linear and nonlinear statistical interactions through defining higher-risk subpopulations. Nevertheless, the use of CART also has some caveats. One of the problems is that the same predictor variable may be selected to split a number of successive levels. As a result, there is a tendency to select predictor variables that can afford more splits in the tree-growing process. To overcome this problem, we used a user-controlled tree-growing approach. With this approach, we determined the priority of predictor variable selection based on the value of the Gini criterion; at the same time, we avoided repeat selections of the same predictor variable in different levels of a tree. This process allows for growing a tree with a reasonable number of levels or branches. The statistical findings based on an overgrown tree with repeat selection of the same variable are often associated with problems of low reliability, where a small alteration in the number of cases may lead to statistically significant changes in risk estimates (*48*).

Historically, research has focused on the critical periods of human development and the ways the effect produced by a given agent might be expected to change when exposure occurs at different times during pregnancy. More recently, evidence shows that critical windows of exposure also encompass preconceptional and postnatal (neonatal, peripubertal/adolescent) time periods (*49*). Our contrasting results for early versus late spontaneous abortions suggest that the developing organism is differentially sensitive to various pesticides at critical periods of development. During the preconception window, damage to spermatozoal DNA can be transmitted to the zygote and may cause early embryo death (*50*). Because the herbicide 2,4-D has been measured in seminal fluid (*27*), there is evidence that at least some pesticides may be delivered to the target site where damage to the spermatozoa could occur.

Although this study was not able to provide any information on dose, it did show that timing of exposure may be as important as dose in characterizing the reproductive toxicity of a chemical product (*4*). Identifying the windows in time when the reproductive system is most sensitive will provide insight into the underlying pathology. Given our results, we recommend that future studies employ a similar statistical methodology to identify potentially toxic agents and mixtures, and examine closely the role of advanced maternal age in the degree of toxicity of an agent. Further epidemiologic research on the reproductive toxicity of glyphosate, carbaryl, the phenoxy acetic acid and triazine herbicides, and thiocarbamate pesticides is warranted.

References and Notes

1. Taha TE, Gray RH. Agricultural pesticide exposure and perinatal mortality in central Sudan. Bull WHO 71:317–321 (1993).
2. Restrepo M, Muñoz N, Day NE, Parra JE, de Romero L, Nguyen-Dinh X. Prevalence of adverse reproductive outcomes in a population occupationally exposed to pesticides in Colombia. Scand J Work Environ Health 16:232–238 (1990).
3. Pastore LM, Hertz-Picciotto I, Beaumont JJ. Risk of stillbirth from occupational and residential exposures. Occup Environ Med 54:511–518 (1997).
4. Pryor JL, Hughes C, Foster W, Hales BF, Robaire B. Critical windows of exposure for children's health: the reproductive system in animals and humans. Environ Health Perspect 108(suppl 3):491–503 (2000).
5. Lemasters GK, Perreault SD, Hales BF, Hatch M, Hirshfield AN, Hughes CL, Kimmel GL, Lamb JC, Pryor JL, Rubin C, et al. Workshop to identify critical windows of exposure for children's health: reproductive health in children and adolescents work group summary. Environ Health Perspect 108(suppl 3):505–509 (2000).
6. Arbuckle TE, Savitz DA, Mery LS, Curtis KM. Exposure to phenoxy herbicides and the risk of spontaneous abortion. Epidemiology 10:752–760 (1999).
7. Savitz DA, Arbuckle T, Kaczor D, Curtis KM. Male pesticide exposure and pregnancy outcome. Am J Epidemiol 146:1025–1036 (1997).
8. Curtis KM, Savitz DA, Weinberg CR, Arbuckle TE. The effect of pesticide exposure on time to pregnancy. Epidemiology 10:112–117 (1999).
9. Arbuckle TE. The Ontario Farm Family Health Study: Development of Survey Instruments and Pilot Study [PhD Thesis]. Chapel Hill, NC:University of North Carolina, 1994.
10. Kline J, Stein Z. Spontaneous abortion (miscarriage). In: Perinatal Epidemiology (Bracken MB, ed). New York: Oxford University Press, 1984;23–51.
11. Breiman L, Friedman J, Olshen R, Stone C. Classification and Regression Tree. Belmont, CA: Wadsworth International Group, 1984.
12. Crichton NJ, Hinde JP, Marchini J. Models for diagnosing chest pain: is CART helpful? Stat Med 16:717–727 (1997).
13. Zhang H, Bracken M. Tree-based risk factor analysis of preterm delivery and small-for-gestational-age birth. Am J Epidemiol 141:70–78 (1995).
14. Zhang H, Bracken MB. Tree-based, two-stage risk factor analysis for spontaneous abortion. Am J Epidemiol 144:989–996 (1996).
15. SPSS Inc. AnswerTree 2.0 User's Guide, Chicago:SPSS Inc, 1998;197.
16. SAS Institute. SAS/STAT User's Guide, Version 7-1, Vol. 3. Cary, NC:SAS Institute Inc., 1999.
17. Källen, B. Epidemiology of Human Reproduction. Boca Raton, FL:CRC Press, 1988.
18. Warburton D, Stein Z, Kline J, Susser M. Chromosome abnormalities in spontaneous abortion: data from the New York City study. In: Human Embryonic and Fetal Death (Porter IH, Hook EB, eds). New York: Academic Press, 1980;261–287.
19. Osborn JF, Cattaruzza MS, Spinelli A. Risk of spontaneous abortion in Italy, 1978–1995, and the effect of maternal age, gravidity, marital status, and education. Am J Epidemiol 151:98–105 (2000).
20. Nybo Andersen A-M, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. Br Med J 320:1708–1712 (2000).
21. Stein Z, Susser M. The risks of having children in later life: social advantage may make up for biological disadvantage. Br Med J 320:1681–1682 (2000).
22. Arbuckle TE, Sever LE. Pesticide exposures and fetal death: a review of the epidemiologic literature. Crit Rev Toxicol 28:229–270 (1998).
23. Charles JM, Cunny HC, Wilson RD, Ivett JL, Murli H, Bus JS, Gollapudi B. In vitro micronucleus assays on 2,4-dichlorophenoxyacetic acid and its derivatives. Mutat Res 444:227–234 (1999).
24. Blakley PM, Kim JS, Firneisz GD. Effects of paternal subacute exposure to Tordon 202c on fetal growth and development in CD-1 mice. Teratology 393:237–241 (1989).
25. Lerda D, Rizzi R. Study of reproductive function in persons occupationally exposed to 2,4-dichlorophenoxyacetic acid (2,4-D). Mutat Res 262:47–50 (1991).
26. Carmelli D, Hofherr L, Tomsic J, Morgan RW. A case-control study of the relationship between exposure to 2,4-D and spontaneous abortions in humans. Washington DC:U.S. Forest Service, U.S. Department of Agriculture, 1981.
27. Arbuckle TE, Schrader SM, Cole D, Hall JC, Bancej CM, Turner LA, Claman P. 2,4-Dichlorophenoxyacetic acid residues in semen of Ontario farmers. Reprod Toxicol 13:421–429 (1999).
28. Taets C, Aref S, Rayburn AL. The clastogenic potential of triazine herbicide combinations found in potable water supplies. Environ Health Perspect 106:197–201 (1998).
29. Munger R, Isacson P, Hu S, Burns T, Hanson J, Lynch CF, Cherryholmes K, Van Dorpe P, Hausler WJ Jr. Intrauterine growth retardation in Iowa communities with herbicide-

contaminated drinking water supplies. Environ Health Perspect 105:308–314 (1997).
30. Meisner LF, Belluck DA, Roloff BD. Cytogenetic effects of alachlor and/or atrazine in vivo and in vitro. Environ Mol Mutagen 19:77–82 (1992).
31. Ribas G, Surralles J, Carbonell E, Creus A, Xamena N, Marcos R. Lack of genotoxicity of the herbicide atrazine in cultured human lymphocytes. Mutat Res 416:93–99 (1998).
32. Lioi MB, Scarfi MR, Santoro A, Barbieri R, Zeni O, Salvemini F, Di Berardino D, Ursini MV. Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed *in vitro* to glyphosate, vinclozolin, atrazine, and DPX-E9636. Environ Mol Mutagen 32:39–46 (1998).
33. Kligerman AD, Doerr CL, Tennant AH, Zucker RM. Cytogenetic studies of three triazine herbicides. I. In vitro studies. Mutat Res 465:53–59 (2000).
34. Cummings AM, Rhodes BE, Cooper RL. Effect of atrazine on implantation and early pregnancy in 4 strains of rats. Toxicol Sci 58:135–143 (2000).
35. Iyer P, Gammon D, Gee J, Pfeifer K. Characterization of maternal influence on teratogenicity: an assessment of developmental toxicity studies for the herbicide cyanazine. Regul Toxicol Pharmacol 29:88–95 (1999).
36. Lioi MB, Scarfi MR, Santoro A, Barbieri R, Zeni O, Di Berardino D, Ursini MV. Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures *in vitro*. Mutat Res 403:13–20 (1998).
37. Rank J, Jensen AG, Skov B, Pedersen LH, Jensen K. Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, *Salmonella* mutagenicity test, and Allium anaphase-telophase test. Mutat Res 300:29–36 (1993).
38. Davis JR, Brownson RC, Garcia R, Bentz BJ, Turner A. Family pesticide use and childhood brain cancer. Arch Environ Contam Toxicol 24:87–92 (1993).
39. Mathur A, Bhatnagar P. A teratogenic study of carbaryl in Swiss albino mice. Food Chem Toxicol 29:629–632 (1991).
40. Rao BV, Srinivas N, Rao PVV Prasada. Clastogenicity of captan and zineb in *Allium meristem* assay. J Environ Biol 21:157–160 (2000).
41. Lieberman AD, Craven MR, Lewis HA, Nemenzo JH. Genotoxicity from domestic use of organophosphate pesticides. J Occup Environ Med 40:954–957 (1998).
42. Padungtod C, Hassold TJ, Millie E, Ryan LM, Savitz DA, Christiani DC, Xu X. Sperm aneuploidy among Chinese pesticide factory workers: scoring by the FISH method. Am J Ind Med 36:230–238 (1999).
43. Burruel VR, Raabe OG, Overstreet JW, Wilson BW, Wiley LM. Paternal effects from methamidophos administration in mice. Toxicol Appl Pharmacol 165:148–157 (2000).
44. Weinberg CR. Toward a clearer definition of confounding. Am J Epidemiol 137:1–8 (1993).
45. Hosmer DW, Lemeshow S. Applied Logistic Regression. New York:John Wiley & Sons, 1989;74.
46. Greenland S. Modeling and variable selection in epidemiologic analysis. Am J Public Health 79:340–349 (1989).
47. Swan GE, Carmelli D, LaRue A. Performance on the digit symbol substitution test and 5-year mortality in the Western Collaborative Group Study. Am J Epidemiol 141:32–40 (1995).
48. Loh W-Y, Shih Y-S. Split selection methods for classification trees. Statistica Sinica 7:815–840 (1997).
49. Selevan SG, Kimmel CA, Mendola P. Identifying critical windows of exposure for children's health. Environ Health Perspect 108(suppl 3):451–455 (2000).
50. Hales BF, Robaire B. Paternally mediated effects on development. In: Handbook of Developmental Toxicology (Hood RD, ed). Boca Raton, FL:CRC Press, 1996;91–107.

Pesticide Data Program

Annual Summary, Calendar Year 2011

United States
Department of
Agriculture

Agricultural
Marketing
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Science and
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Visit the program Web site at: www.ams.usda.gov/pdp

February 2013

USDA Agricultural Marketing Service

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February 2013

Dear Reader:

I am pleased to present the Pesticide Data Program's (PDP) 21st Annual Summary for calendar year 2011. The U.S. Department of Agriculture implemented the PDP in 1991 to test food commodities for pesticide residues. The data produced by the PDP are used to estimate consumer dietary exposure to pesticides and the relationship of those exposures to science-based standards of safety. This report shows that overall pesticide residues found on foods tested are at levels below the tolerances (maximum legal residue levels) set by the U.S. Environmental Protection Agency (EPA).

Using a rigorous statistical approach to sampling along with the most current laboratory methods, the PDP tests a wide variety of domestic and imported foods. Foods tested include fresh and processed fruit and vegetables, soybeans, eggs, dairy products, and water.

The 1996 Food Quality Protection Act (FQPA) directs the Secretary of Agriculture to collect pesticide residue data on foods that are highly consumed, particularly by infants and children. The FQPA also established a strict health-based standard for a "reasonable certainty of no harm" for pesticide residues in food to ensure consumer protection from unacceptable pesticide exposure. The EPA uses the PDP data as a critical component for dietary assessments of pesticide exposure, a critical step to verify that all sources of exposure to pesticides meet the safety standards set by the 1996 FQPA.

The PDP is not designed for enforcement of EPA tolerances. However, we inform the U.S. Food and Drug Administration if residues detected exceed the EPA tolerance or have no EPA tolerance established. In 2011, residues exceeding the tolerance were detected in 0.27 percent (32 samples) of the total samples tested (11,894 samples). Residues with no established tolerance were found in 3.4 percent (399 samples) of the total samples tested. The data reported by PDP corroborate that residues found in fruit and vegetables are at levels that do not pose risk to consumers' health (i.e., are safe according to EPA).

The PDP works with cooperating State agencies that are responsible for sample collection and analysis. Thirteen states participated in the program during 2011: California, Colorado, Florida, Maryland, Michigan, Minnesota, Montana, New York, North Carolina, Ohio, Texas, Washington, and Wisconsin. These States represent all regions of the country and more than half of the U.S. population.

For more information please visit our website at www.ams.usda.gov or the EPA at http://www.epa.gov/pesticides/food.

Sincerely,

David R. Shipman

David R. Shipman
Administrator

Acknowledgements

The States participating in the Pesticide Data Program (PDP) deserve special recognition for their contributions to the program. The dedication and flexibility of sample collectors allow the Agricultural Marketing Service (AMS) to adjust sampling protocols when responding to changing trends in commodity distribution and availability. PDP acknowledges the contributions of the State laboratories, the U.S. Department of Agriculture's (USDA) AMS National Science Laboratory, and the USDA Grain Inspection, Packers and Stockyards Administration Laboratory in providing testing services to the program, and the USDA National Agricultural Statistics Service for providing statistical support. PDP also acknowledges the exceptional support of the Health Effects Division staff of the U.S. Environmental Protection Agency, Office of Pesticide Programs, in helping set the direction for PDP.

Data presented in this report are the latest available and were collected and processed through the efforts of the following organizations:

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APPENDIX C. DISTRIBUTION OF RESIDUES BY PESTICIDE IN SOYBEANS

Pesticide	Pest. Type	Number of Samples	Samples with Detections	% of Samples with Detects	Range of Values Detected, ppm	Range of LODs, ppm	EPA Tolerance Level, ppm
Acephate	I	300				0.040 ^	1.0
Acetochlor	H	300				0.003 ^	1.0
Alachlor	H	300				0.001 ^	1.0
Aldicarb	I	300				0.010 ^	0.02
Aldicarb sulfone	IM	300				0.003 ^	0.02
Aldicarb sulfoxide	IM	300				0.010 ^	0.02
Aminomethylphosphonic acid (AMPA)	HM	300	287	95.7	0.26 - 20	0.25 ^	NA
Azoxystrobin	F	280	10	3.6	0.001 - 0.003	0.001 ^	0.5
Bendiocarb	I	279				0.002 ^	NT
Benoxacor	S	300				0.003 - 0.010	NT
Boscalid	F	280				0.002 - 0.005	0.1
Carbaryl	I	300				0.003 ^	4.0
Carbendazim (MBC)	F	300				0.002 ^	NT
Carbofuran	I	300				0.003 ^	1.0
Carboxin	F	300	4	1.3	0.001 - 0.002	0.001 ^	0.2
Chlorimuron ethyl	H	239				0.010 ^	0.05
Chlorpyrifos	I	300	8	2.7	0.003 - 0.005	0.003 ^	0.3
Clofencet	P	300				0.030 ^	30.0
Clomazone	H	300				0.003 ^	0.05
Clothianidin	I	300				0.003 ^	0.02
Cyfluthrin	I	300				0.010 ^	0.03
Cyhalothrin, Total (Cyhalothrin-L + R157836 epimer)	I	300	1	0.3	0.010 ^	0.010 ^	0.01
Cypermethrin	I	300				0.025 ^	0.05
Cyproconazole	F	300				0.005 ^	0.05
Deltamethrin (includes parent Tralomethrin)	I	300				0.015 - 0.050	0.1
Difenoconazole	F	280				0.003 ^	0.15
Dimethenamid	H	300				0.001 ^	0.01
Dimethoate	I	300				0.008 ^	0.05
Disulfoton	I	300				0.006 ^	NT
Disulfoton sulfone	IM	300				0.030 ^	NT
Disulfoton sulfoxide	IM	231				0.004 - 0.012	NT
Epoxiconazole	F	300				0.004 ^	NT
EPTC	H	255				0.003 - 0.010	NT
Esfenvalerate	I	300				0.010 ^	0.25
Ethalfluralin	H	300				0.001 ^	0.05
Fenarimol	F	300				0.031 ^	NT
Fenoxaprop ethyl	H	300				0.002 ^	0.05
Fenpropathrin	I	300				0.004 - 0.014	NT

Pesticide	Pest. Type	Number of Samples	Samples with Detections	% of Samples with Detects	Range of Values Detected, ppm	Range of LODs, ppm	EPA Tolerance Level, ppm
Fluazifop butyl	H	300				0.003 ^	2.5
Fludioxonil	F	300				0.008 ^	0.01
Flumetsulam	H	300				0.010 ^	0.05
Fluquinconazole	F	240				0.014 ^	NT
Fluridone	H	294	1	0.3	0.001 ^	0.001 ^	0.1
Flutriafol	F	300				0.004 ^	0.35
Glyphosate	H	300	271	90.3	0.26 - 18.5	0.25 ^	20.0
Hydroprene	R	300				0.021 ^	0.2
3-Hydroxycarbofuran	IM	300				0.003 ^	1.0
5-Hydroxythiabendazole	FM	300				0.003 ^	0.1
Imazaquin	H	300				0.003 ^	0.05
Imidacloprid	I	300				0.003 ^	3.5
Indoxacarb	I	280				0.027 ^	0.80
Lactofen	H	298				0.009 ^	0.01
Linuron	H	300				0.003 - 0.010	1.0
Malathion	I	300	11	3.7	0.006 - 0.20	0.006 ^	8
Malathion oxygen analog	IM	300				0.003 ^	8
Metalaxyl/Mefenoxam *	F	300				0.002 - 0.007	1.0
Methamidophos	I	300				0.030 ^	NT
Methomyl	I	300				0.003 - 0.010	0.2
Methoxyfenozide	I	300	2	0.7	0.007 - 0.063	0.003 ^	1.0
Metolachlor	H	300				0.002 ^	0.20
Metribuzin	H	300				0.007 ^	0.3
Myclobutanil	F	300				0.002 ^	0.25
Norflurazon	H	300				0.015 ^	0.1
Norflurazon desmethyl	HM	300				0.005 ^	0.1
Omethoate	IM	300				0.004 ^	0.05
Oxadixyl	F	300				0.003 ^	NT
Oxamyl	I	300				0.003 ^	0.1
Oxyfluorfen	H	300				0.002 ^	0.05
Parathion ethyl	I	300				0.004 ^	NT
Parathion methyl	I	300				0.006 ^	0.1
Parathion methyl oxygen analog	IM	300				0.002 ^	0.1
Parathion oxygen analog	IM	300				0.025 ^	NT
Pendimethalin	H	300				0.006 ^	0.1
Permethrin Total	I	300				0.009 ^	0.05
Phorate	I	300				0.004 ^	0.05
Prallethrin	I	300				0.045 ^	1.0
Propetamphos	I	280				0.001 ^	0.1
Propiconazole	F	300				0.003 ^	2.0
Pymetrozine	I	280				0.003 ^	NT
Pyraclostrobin	F	300	20	6.7	0.001 - 0.022	0.001 ^	0.04

Pesticide	Pest. Type	Number of Samples	Samples with Detections	% of Samples with Detects	Range of Values Detected, ppm	Range of LODs, ppm	EPA Tolerance Level, ppm
Pyriproxyfen	I	300				0.032 ^	0.2
Quizalofop ethyl	H	300	2	0.7	0.002 ^	0.002 ^	0.05
Resmethrin	I	300				0.002 ^	3.0
Spinosad A	IM	300				0.003 - 0.010	0.02
Sulfentrazone	H	300				0.015 - 0.050	NT
Tebuconazole	F	300				0.004 ^	0.08
Tetraconazole	F	295	1	0.3	0.008 ^	0.006 ^	0.15
Tetrahydrophthalimide (THPI)	FM	300				0.015 ^	0.05
Thiabendazole	F	300				0.003 ^	0.1
Thiamethoxam	I	300				0.001 ^	0.08
Thifensulfuron methyl	H	300				0.006 - 0.020	1.0
Trifloxystrobin	F	300	2	0.7	0.001 - 0.003	0.001 ^	0.08
Trifluralin	H	300				0.002 ^	0.1

Many of the listed tolerances are the sum of a parent compound and metabolite(s)/isomer(s). The reader is advised to refer to EPA for the complete listing of compounds in tolerance expressions. The cited tolerances apply to 2011 and not to the current year. There may be instances where a tolerance was recently set or revoked that would have an effect on whether a residue is violative or not.

NOTES

^ = Only one distinct detected concentration or LOD value was reported for the pair.

NA = AMPA, a degradation product of glyphosate, is not subject to the food tolerance established for glyphosate. The tolerance applies only to the parent compound.

NT = No tolerance level was set for that pesticide/commodity pair.

* = Metalaxyl and mefenoxam have separate registrations. Mefenoxam is also known as Metalaxyl-M, which is one of the spatial isomers comprising metalaxyl. The spatial isomers of metalaxyl are analytically indistinguishable via multiresidue methods.

Pesticide Types:

F = Fungicide, FM = Fungicide Metabolite
H = Herbicide, HM = Herbicide Metabolite
I = Insecticide, IM = Insecticide Metabolite
P = Plant Growth Regulator
R = Insect Growth Regulator
S = Herbicide Safener

LAMAR S. SMITH, Texas
CHAIRMAN

EDDIE BERNICE JOHNSON, Texas
RANKING MEMBER

Congress of the United States
House of Representatives

COMMITTEE ON SCIENCE, SPACE, AND TECHNOLOGY

2321 RAYBURN HOUSE OFFICE BUILDING

WASHINGTON, DC 20515-6301

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May 4, 2016

The Honorable Gina McCarthy
Administrator
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, D.C. 20460

Dear Administrator McCarthy:

The Committee on Science, Space, and Technology is conducting oversight of U.S. Environmental Protection Agency's (EPA) risk analysis prepared by the Cancer Assessment Review Committee (CARC). According to recent media reports, on April 29, 2016, EPA posted what appears to be the final risk assessment for glyphosate prepared by CARC (the CARC report).[1] The CARC report indicates that glyphosate is "Not Likely to be Carcinogenic to Humans."[2] Press reports indicate that EPA removed this document on May 2, 2016.[3] Subsequently, EPA has asserted that the analysis of glyphosate is not final and that the documents were posted "inadvertently."[4]

The Committee has reviewed the CARC report and point out that it is clearly marked as a "Final Report."[5] The report also contains the signatures of thirteen members of CARC.[6] However, EPA's removal of this report and the subsequent backtracking on its finality raises questions about the agency's motivation in providing a fair assessment of glyphosate – an assessment based on the scientific analysis conducted by CARC. Furthermore, EPA's apparent mishandling of this report may shed light on larger systemic problems occurring at the agency. In order to assist the Committee in its oversight of the EPA's assessment of glyphosate, please

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[1] P.J. Huffstutter, *EPA Takes Offline Report that Says Glyphosate Not Likely Carcinogenic,* Reuters, May 2, 2016, *available at* http://www.reuters.com/article/us-usa-glyphosate-epa-idUSKCN0XU01K.
[2] Evaluation of the Carcinogenic Potential of Glyphosate, Final Report, Cancer Assessment Review Committee, U.S. EPA, Oct. 1, 2015, *available at* http://src.bna.com/eAi.
[3] P.J. Huffstutter, *EPA Takes Offline Report that Says Glyphosate Not Likely Carcinogenic,* Reuters, May 2, 2016, *available at* http://www.reuters.com/article/us-usa-glyphosate-epa-idUSKCN0XU01K.
[4] *Id.*
[5] Evaluation of the Carcinogenic Potential of Glyphosate, Final Report, Cancer Assessment Review Committee, U.S. EPA, Oct. 1, 2015, *available at* http://src.bna.com/eAi.
[6] *Id.*

provide all documents and communications from January 1, 2015, to the present, referring or relating to the CARC report on glyphosate by 5:00 p.m. on May 18, 2016.

The Committee on Science, Space, and Technology has jurisdiction over environmental and scientific programs and "shall review and study on a continuing basis laws, programs, and Government activities" as set forth in House Rule X.

The Committee requests that you provide the requested documents and information, in electronic format. An attachment to this letter provides details on producing documents to the Committee.

If you have any questions about this request, please contact Joseph Brazauskas or Taylor Jordan of the Science, Space, and Technology Committee staff at 202-225-6371. Thank you for your attention to this matter.

Sincerely,

Lamar Smith

Lamar Smith
Chairman

cc: The Honorable Eddie Bernice Johnson, Ranking Minority Member, House Committee on Science, Space and Technology

Moore-Love, Karla

From: Alexander Krokus <alexander.krokus@pcc.edu>
Sent: Saturday, June 04, 2016 5:15 PM
To: Moore-Love, Karla
Subject: Communication to City Council: Glyphosate (06-08-2016)
Attachments: TestimonyGlyphosate-AlexanderKrokus.pdf

June 4, 2016

City of Portland Council Clerk
Karla Moore-Love
1221 SW 4th Avenue, Room 130
Portland, OR 97204

Re: Communication to City Council: Glyphosate (06-08-2016)

Dear Karla Moore-Love,

I hope that you are enjoying your weekend. I've attached a PDF of my finalized testimony to this email. I will also be providing hard copies of relevant credible scientific studies, statistical charts, and graphs, as additional supplements to support my testimony.

Thank you once again for enabling this communication.

See you next week.

Sincerely,

Alexander Krokus
5555 N. Wilbur Ave.
Portland, OR 97217
alexander.krokus@pcc.edu
akrokus@pdx.edu

5

Moore-Love, Karla

From: Alexander Krokus <alexander.krokus@pcc.edu>
Sent: Wednesday, May 18, 2016 1:07 PM
To: Moore-Love, Karla
Subject: Request to Address Communication to Council: Alexander Krokus
Attachments: FinalTestimonyAlexanderKrokus.pdf

May 18, 2016

City of Portland Council Clerk
Karla Moore-Love
1221 SW 4th Avenue, Room 130
Portland, OR 97204

Re: Request to Address Communication to Council

Dear Karla Moore-Love,

I genuinely appreciate your time reviewing this testimony. This communication has been created as a result of the initial efforts of Susan Moray, and for a recently created group of concerned community members. We are attempting to provide prudent rationale, to help eliminate the use of the herbicide glyphosate, in all public parks located in the city of Portland.

I've attached a PDF of my testimony, along with credible sources to this email. If you have any questions, or necessitate any other information, please do not hesitate to ask.

Here is a link to a local news story pertaining to the petition Susan Moray created. It has already obtained over 18,000 signatures.

http://katu.com/news/local/petition-asks-portland-parks-and-rec-to-stop-use-of-controversial-weed-killer

Thank you immensely for you time once again, and the opportunity to have our voices heard.

Sincerely,

Alexander Krokus
5555 N. Wilbur Ave.
Portland, OR 97217
alexander.krokus@pcc.edu

May 10, 2016

RE: Logical Rationale for the Elimination of the Spraying of All Glyphosate based Herbicides, in Parks Managed by Portland Parks & Recreation.

Glyphosate is a systemic herbicide. It penetrates and encompasses the entire plant that it is infecting, and will eradicate any additional plants which are not genetically engineered to resist it. Glyphosate's chemical effect is primarily to block enzymes that plants necessitate to exist, and it also reduces their production of amino acids and vital proteins. (Hoagland et al.) In 1985, acting out on the scientific discoveries of tumor formations on mice, the EPA originally classified glyphosate as *"possibly carcinogenic to humans"*, placing this chemical in *"Group C"*. Six years later, the EPA oddly decided to alter its classification of glyphosate by moving it to *"Group E"*, stating that it is *"non-carcinogenic to humans."* (IARC)

During 2014, an international Advisory Group containing senior scientists and government officials, advised the World Health Organization to evaluate dozens of pesticides, to determine if there is any potential association with these chemicals, that could produce adverse human health effects.

On March 20, 2015 the International Agency for Research on Cancer, which is part of the World Health Organization, which represents 194 member states internationally, declared glyphosate as a *"Group 2A"* carcinogen. This category is defined as *"probably carcinogenic to humans"*. Their justification for this classification was based on *"convincing evidence that glyphosate cause cancer in laboratory animals"*, and by evaluating significant findings from EPA reports, concluding that *"there is sufficient evidence of carcinogenicity"* that has been clearly documented during animal laboratory studies. The IARC Working Group also observed *"DNA and chromosomal damage in human cells"* transpiring during this unbiased scientific research.

Glyphosate has the *"highest global production of all herbicides"*. The agricultural use of this product has increased exponentially alongside the introduction of genetically modified crops, which are scientifically formulated to become resistant to the negative effects of glyphosate. The presence of glyphosate has been detected in the *"air during spraying, in water, and in food"*, all across the world. (WHO) A study was conducted in late 2010 in Berlin, Germany that found glyphosate visible in human urine. The urine samples were provided by *"city workers, journalists, and lawyers, who had no direct contact with glyphosate"*. They were examined for contamination by a research team at the University of Leipzig, and all of the subjects tested positive for glyphosate. These were values ranging from *"0.5 to 2 ng glyphosate*

per ml urine (drinking water limit: 0.1 ng / ml)". Not one single examinee experienced any direct contact with agriculture prior to the testing. (Brandli et al.)

The IARC *"Group 2A"* classification lists numerous carcinogens that have been labeled as extremely dangerous. Some of these substances are even illegal, nationally, and internationally. *"Lead compounds (inorganic), petroleum refining, polybrominated biphenyls (PBB's), human papillomavirus (HPV), Dichlorodiphenyltrichloroethane (DDT's), and Androgenic (anabolic) steroids"* are listed in the same carcinogenic category as glyphosate.

The IARC's recommendations are based on *"scientific evidence based on a comprehensive view of the scientific literature"*. Unfortunately, the implementation of enforcing these ethical standards are the responsibility of individual governments, and other international organizations, who must establish the regulations, or legislation to act upon.

International experts of toxicology have demonstrated glyphosate's ability to impede the progression of *"puberty, body development, the hormonal production of testosterone, estradiol and corticosterone."* Glyphosate has also been scientifically documented to significantly alter testicular morphology. (Bernardi et al.)

After conducting extensive research for over a decade, Denmark scientists discovered that glyphosate can be filtered through various soil types, by the action of raining, which will eventually send these toxic herbicides into drains, that eventually lead into rivers, and then into oceans. (Brusch et al.) This particular study led to Denmark enacting a ban on glyphosate being used on paved surfaces, because of subsequent *"urban runoff"* poisoning their waterways. (Franzen et al.)

A study performed by the Bureau of Reproductive and Child Health, which is located in Ottawa, Canada, observed an associated risk linked to glyphosate that produced spontaneous abortions in an Ontario farm population. Their results revealed that "*among older women exposed to glyphosate, the risk for spontaneous abortion was three times higher than women of the same age who were not exposed to this active ingredient."* (Arbuckle et al.) Multiple international experts in toxicology also consider the genotoxicity of glyphosate to be associated in producing higher risks of obtaining childhood brain cancer. (Bentz et al.)

The chair of the federal House of Representatives Committee on Science, Space, & Technology has recently launched an investigation on May 4, 2016, regarding the EPA's immensely anticipated report relating to potential negative human health effects, specifically pertaining to glyphosate. The EPA released a *"Final Report"* from the EPA's Cancer Assessment Review Committee, that was signed by 13 scientists, for the House Committee to review, and then the EPA immediately removed the data from the system. House Committee on Science, Space, & Technology chair Lamar Smith, is demanding that the EPA produce every single document and scientific study, and all communications records related to glyphosates, that transpired from January 1, 2015 to present, to the committee. The deadline for the EPA to provide this critical data is May 18, 2016. The Committee on Science, Space, and Technology has complete *"jurisdiction over environmental and scientific programs"* and the EPA. (Smith)

These EPA documents are being reviewed to determine if glyphosates can be considered as a safe practice in light of recent scientific research. This is the first time that the EPA *"fully analyzed the threats posed by glyphosate"* since *"1993"*. (Center for Biological Diversity)

Our primary obstacle in providing adequate protection for residents living in the Portland metropolitan area, and all across the nation, is that we are relying on the EPA as our sole source for regulating pesticides and herbicides. The EPA represents a country with only 4.4% of the world's population, the World Health Organization represents 194/196 nation-states. On May 6, 2016, the Monsanto Company produced a peculiar statement on their twitter account. It was regarding this highly meaningful and suspiciously unrevealed EPA study, which stated *"Have you heard glyphosate causes cancer? The EPA disagrees."* (Monsanto Company) Why does the Monsanto Company have any access to this highly confidential information, that the House Committee on Space, Science, and Technology, who provides oversight to the EPA, cannot even obtain?

Unbiased, and non-industry funded credible science, must be the determining factor when making decisions to protect the health and security of humankind. This is how the World Health Organization made their logical and ethical decision on glyphosates, so why can't we be rational thinking human beings too?

Thank you for sincerely for spending the time to read this. It is genuinely appreciated.

Alexander Krokus
5555 N. Wilbur Ave.
Portland, OR 97217
alexander.krokus@pcc.edu

Works Cited

Arbuckle, Tye, Zhiqiu Lin, Leslie S. Mery. *"An Exploratory Analysis of the Effect of Pesticide Exposure on the Risk of Spontaneous Abortion in an Ontario Farm Population"*. Bureau of Reproductive and Child Health, Population and Public Health Branch, Health Canada, Ottawa, Ontario, Canada; Cancer Bureau, Population and Public Health, Health Canada, Ottawa, Ontario, Canada. Environmental Health Perspectives Vol. 109:8. August 2001.

Bentz, BJ, RC Brownson, JR Davis, R Garcia, A Turner. *"A Family Pesticide use and Childhood Brain Cancer"* Arch Environmental Contamination Toxicology 24:87-92. 1993.

Bernardi, MM, PV Furtado, CA Oliveira, MA Romano, RM Romano. *"Prepubertal Exposure to Commercial Formulation of the Herbicide Glyphosate Alters Testosterone Levels and Testicular Morphology."* National Center for Biotechnology Information. U.S. National Library of Medicine. Arch Toxicology 84(4):309-17. 12 Dec 2009.

Brandli, Dirk, Sandra Reinacher. *"Herbicides Found in Human Urine"*. Delinat Institute for Ecology and Climate-Farming. Viticulture Ecology Climate Farming. Ithaka Journal. Germany. Jan 2012.

Brusch, W, V Ernstsen, L Gudmundsson, R Grant, RK Juhler, F Plauborg, P Nyegaard, P Olsen. *"The Danish Pesticide Leaching Assessment Programme Monitoring Results May 1999-June 2009"*. Geological Survey of Denmark and Greenland, Ministry of Climate and Energy and Faculty of Agriculture Sciences. 2010.

Center for Biological Diversity. *"EPA Uses Industry-Funded Studies to Determine Glyphosate Does Not Cause Cancer."* Portland, Oregon. 2 May 2016.

Franzen, I, AC Grundy, C Kempenaar, P Kristoffersen, J Raisio, H Schroeder, A Verschwele, L Zarina. *"A Review of Pesticide Policies and Regulations for Urban Amenity Areas in Seven European Countries"*. Weed Research 48(3). 2008.

Monsanto Company. *"Have you Heard Glyphosate Causes Cancer? The EPA Disagrees. monsanto.info/26MNNB3"*. Twitter. @MonsantoCo. 2 May 2016.

Smith, Lamar S. *"The Honorable Gina McCarthy: Administrator - U.S. Environmental Protection Agency"*. Congress of the United States, House of Representatives. Committee on Science, Space, and Technology. Washington, D.C. 4 May 2016.

World Health Organization. *"IARC Monographs Volume 112: Evaluation of Five Organophosphate Insecticides and Herbicides"*. International Agency for Research on Cancer. Lyon, France. 20 March 2015.

607

Request of Alexander Krokus to address Council regarding eliminate the use of the herbicide glyphosate in Portland parks (Communication)

JUN 08 2016

PLACED ON FILE

Filed MAY 31 2016

MARY HULL CABALLERO
Auditor of the City of Portland

By _____
Deputy

COMMISSIONERS VOTED AS FOLLOWS:		
	YEAS	NAYS
1. Fritz		
2. Fish		
3. Saltzman		
4. Novick		
Hales		