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EXPERIMENTAL PROJECT ON THE SAFETY AND THE OPTIMIZATION OF THE USE OF GLYPHOSATE-BASED HERBICIDES IN THE GRAIN SECTOR.

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Abstract

INTRODUCTION: Glyphosate is the most widely applied pesticide worldwide and it is an active ingredient of all glyphosate-based herbicides (GBHs), including in the formulation "Roundup" . It is unclear if the glyphosate present in ground water can be absorbed and translocated in different parts of the pants, particularly wheat plants. This indeed represents an important aspect for productivity (being this a powerful herbicide) and organic certification of the products (the use of glyphosate is not admitted in organic farming and the ubiquitous contamination of glyphosate in water might in theory affect the level of glyphosate in the plants). Overall, epidemiological, *in vivo* and *in vitro* studies available in literature present conflicting findings on the safety of glyphosate.

METHODS: The work performed for this PhD thesis aimed to experimentally test the root absorption and the eventual translocation of the glyphosate herbicide in the different parts of the wheat plant (Triticum durum) starting from ground water. Furthermore we aimed to experimentally test the effects of the exposure to GBHs at doses of glyphosate considered to be "safe", the US ADI of 1.75 mg/kg bw/day, defined as the chronic Reference Dose (cRfD) determined by the US EPA, in in vivo models (Sprague-Dawley rats) and in vitro models (Caco2 and L929).

RESULTS: All the experimental absorption studies on wheat plants performed have given negative results in terms of the presence of glyphosate or AMPA in the grain of durum wheat. On the other hand the experimental safety studies on in vitro and in vivo models highlighted different effects at doses currently considered safe for humans and with no effects in animals.

CONCLUSION: Overall the integration of the findings from absorption in plants and safety studies will serve as solid evidence-base for risk assessment and productive strategies for agriculture.

1. INTRODUCTION

1.1 Glyphosate-Based Herbicides: Global Use and Burden of Exposure

Glyphosate [IUPAC chemical name N-(phosphonomethyl)glycine] is the most widely applied pesticide worldwide and it is an active ingredient of all glyphosate-based herbicides (GBHs), including in the formulation "Roundup" (Benbrook 2016; Myers et al. 2016). It is mainly marketed as a *broad-spectrum* systemic *herbicide* and crop desiccant (Smith and Oehme 1992). The substance glyphosate, as an amino acid analogue of glycine, was initially synthesizedin 1950 by a Swiss chemist, Henri Martin, at the pharmaceutical company Cilag. Its herbicidal properties were not discovered for another 20 years. Since glyphosate was patented in 1974 by Monsanto as a herbicide, approximately 9.4 million tons of GBHs have been sprayed, nearly half a pound of glyphosate on every cultivated acre of land globally. In terms of production of active ingredient, Asia-Pacific represents the largest regional supplier of glyphosate worldwide. In 2016, China contributed the largest share in Asia Pacific, and is likely to remain a dominant market for many years. The United State trails behind the Asia-Pacific market in the production of GBHs. Latin America, Middle East and Africa are expected to grow in terms of use at a significant rate during the forecast period for 2017-2025 (Research 2017). Production and use of glyphosate have risen dramatically with the introduction in 1996 of GM - genetically modified - glyphosate tolerant crop varieties. In the United States (US) it is contained in over 750 products, in particular herbicides used for intensive GM – genetically modified – crops growing that have built-in tolerance to glyphosate, but also in other products for agriculture, forestry, urban, and home applications (IARC 2015). In 2015, 89%

of corn, 94% of soybeans, and 89% of cotton cropped in the US were genetically modified to be glyphosate-tolerant (USDA 2015). Only a few data on the use of individual pesticides are available for certain countries in the European Union (EU), making it difficult to find out how much glyphosate is being used by farmers (Garthwaite et al. 2015). However, surveys in individual countries give some indication. Glyphosate is the top ranked herbicide in United Kingdom arable crop production (Garthwaite et al. 2010). In Denmark, glyphosate accounts for 35% of all pesticides used in agricultural production (DEPA 2009). In Germany, it has been estimated that glyphosate is used on 4.3 million hectares (39%) of agricultural land each year, with nearly two thirds applied to just 3 crops - oilseed rape, winter wheat and winter barley (Steinmann et al. 2012). The EU has a strict regulation regarding the planting of GM crops (Directive EU 2015/412) (EU 2015) and GBHs are mainly applied to cereals for post-harvest desiccation purposes (wheat, rye, triticale, barley and oats), oilseeds (rapeseed, mustard seed and linseed), orchards and vineyards (EFSA 2015).

The massive and increasing use of GBHs leads to a global burden of occupational exposures in manufacturing workers and GBH applicators (farmers), as well as increasing exposures in the general population, as demonstrated by environmental contamination from glyphosate residues found in air (Majewski et al. 2014), groundwater (Battaglin et al. 2014; ISPRA 2016), drinking-water (Rendón-von Osten and Dzul-Caamal 2017), crops (Cuhra 2015; USDA 2013), food (EFSA 2016; PRIF 2016) and animal feed (Mesnage et al. 2015). The maximum concentration of glyphosate found out of 185 monitoring sites in Italian groundwater was 1.08 μg (ISPRA 2016). It is unclear if the glyphosate present in ground water can be absorbed and translocated in different parts of the pants,

particularly wheat plants. This indeed represents an important aspect for productivity (being this a powerful herbicide) and organic certification of the products (the use of glyphosate is not admitted in organic farming and the ubiquitous contamination of glyphosate in water might in theory affect the level of glyphosate in the plants).

Microbial biodegradation of glyphosate occurs in soil, aquatic sediment and water. The main pathway of biodegradation of glyphosate appears to be by splitting the C–N bond to produce aminomethylphosphonic acid (AMPA), the major microbial metabolite (WHO 2005). In humans, the main exposure routes to glyphosate are inhalation and dermal exposure in the occupational setting and consumption of water and food for the general population (WHO 2005). The results of oral studies with [^{14}C] glyphosate in rats, rabbits and goats indicate that absorption from the gastrointestinal tract is incomplete and amounts to up to 30% of the dose (Brewster et al. 1991; Colvin and Miller 1973; Powles 1994). The most relevant routes of excretion following oral administration of glyphosate [^{14}C] are feces (70-80%) and urine (20-30%) (NTP 1992). In rats, after a single oral administration of [^{14}C] glyphosate, almost all radioactivity was detected in urine and feces, and the radiolabeled detected chemical was present as the unchanged parent compound (Davies 1996a, b, c). Elimination through exhaled air was very low. AMPA was the only metabolite detected, accounting for only 0.2–0.3% of the applied dose of [^{14}C] glyphosate (Howe et al. 1988). The limited data currently available on glyphosate pharmacokinetics in vertebrates are insufficient to predict transport and fate of glyphosate in different mammalian tissues, organs and fluids in the body, and to determine whether or where bioaccumulation occurs, although animal metabolism studies indicate kidney and

liver as target tissues (Myers et al. 2016).

1.2 Safety Profile of Glyphosate and its Formulations

The possible effects of GBHs on human health is the topic of intense public debate, for both its potential carcinogenic and non-carcinogenic effects, including endocrine disruption, neurotoxicity, developmental and reproductive toxicity, which might occur even at doses much lower than the ones considered for risk assessment, in particular during sensitive periods of life (such as fetal development) (ECHA 2017; EFSA 2015; IARC 2015; Shehata et al. 2013). Glyphosate, as the pure active substance, and GBHs may not be quite the same from the toxicological standpoint. Glyphosate formulations contain a number of so-called 'inert' ingredients or adjuvants to facilitate the uptake by plants, most of which are patented and not publicly known (in many countries the law does not require a full disclosure of pesticide ingredients). GBHs that contain surfactants and adjuvants might act differently than glyphosate alone (Landrigan and Benbrook 2015; Mullin et al. 2016). In fact, adjuvants might potentiate the toxic effects of glyphosate (Coalova et al. 2014; Defarge et al. 2016; Mesnage et al. 2013; Williams et al. 2000). Epidemiological evidence of GBH effects on reproductive and developmental health outcomes is too limited to draw conclusions The Ontario Farm Family Health Study (OFFHS), detected a significant association between preconception exposure to pesticide products containing glyphosate and increased risk of spontaneous abortion (Arbuckle et al. 2001; Savitz et al. 1997). A small recent study found a significant correlation between urine glyphosate levels in pregnant women and shorter gestational length

(Parvez et al. 2018). However, most existing epidemiological studies present methodological limitations, lack of measurement of internal glyphosate exposure (urine and/or blood levels), no investigation on the biological plausibility of findings and sometimes also inadequate control of confounders (de Araujo et al. 2016; Parvez et al. 2018). Overall, both in vitro and in vivo studies available in literature present conflicting findings; glyphosate alone or, mainly, GBHs exposure might be related to adverse developmental or reproductive effects, albeit in many studies only very high dose levels were tested. Effects have been tested mainly in male rats, from different strains, at different lifestages and using different endpoints. Furthermore, it is not clear whether the possible adverse effects might be due to endocrine disrupting or to other mechanisms (Mesnage et al. 2017). Interpretation of the data, particularly for measurement of circulating hormones, should also take into account that potential biological variability could be introduced by different animal models and study design together with not comparable pre-analytical conditions (Bielohuby et al. 2012).

In March 2015 the World Health Organization's International Agency for Research on Cancer (IARC) published an extensive review of the published peer-reviewed epidemiologic, toxicologic and genetic literature on glyphosate, independent of influence by the pesticide manufacturing industry, and concluded that glyphosate is "probably carcinogenic to man" (Category 2A) (IARC, 2015). In November 2015 the EFSA deemed glyphosate "unlikely to pose a cancer risk for man". That conclusion was based on a glyphosate renewal assessment report (RAR) presented in January 2014 by the Federal German Institute for Risk Assessment (Bundesinstitut für Risikobewertung, BfR) (EFSA 2015). The EFSA

and RAR review groups included scientists that did not disclose their names and financial interests and relied also on previously unpublished, non-peer-reviewed reports generated by industry (EFSA 2018). In March 2017 the European Union (EU) appointed the European Chemicals Agency (ECHA) to look into the issue of glyphosate toxicity. The ECHA's Risk Assessment Committee analysed an enormous amount of scientific data and concluded that "the scientific evidence so far available does not satisfy the criteria for classifying glyphosate as carcinogenic, mutagenic or toxic for reproduction." (ECHA 2018). According to the ECHA, glyphosate may cause grave damage to the eyes and be toxic to aquatic organisms with long-term effects. In 2019 a US federal health agency, the Agency for Toxic Substances and Disease Registry (ATSDR), housed in the Centers for Disease Control and Prevention (CDC), published a scientific review and assessment that identified both cancer and non-cancer risks from glyphosate and GBHs. The ATSDR 2019 report clearly lays out the vast array of scientific evidence linking both pure glyphosate (rodent studies) as well as formulated GBH products (in human epidemiologic studies) like Roundup to cancer. The current approval of glyphosate license in EU will expire on 15 December 2022 and the process for renewal must begin in December 2019.

2. AIMS

The work performed for this PhD thesis aimed to experimentally test the root absorption and the eventual translocation of the glyphosate herbicide in the different parts of the wheat plant (*Triticum durum*) starting from ground water. Furthermore we aimed to experimentally test the effects of the exposure to GBHs

at doses of glyphosate considered to be “safe”, the US ADI of 1.75 mg/kg bw/day, defined as the chronic Reference Dose (cRfD) determined by the US EPA, in in vivo models (Sprague-Dawley rats) and in vitro models (Caco2 and L929).

3. MATERIALS AND METHODS

3.1 Experimental Absorption Studies on Wheat Plants

The objective was to simulate, through two pot tests and a radiolabeled experiment, the possible absorption and translocation of the glyphosate to the grain starting from the groundwater.

The test was performed considering the following aspects:

- The administration of glyphosate has been done trying to simulate what happens in the soil in contact with the aquifer. The pots were kept in immersion in control water or treated water, simulating a height of about 20 cm (values easily found in the Po Valley during the wheat growth phases);

- As reported in the literature (Gomes et al. 2015), a phosphatic fertilizer increases the efficiency of the treatment with glyphosate, increasing its absorption through the roots. Therefore, following the transplantation of the wheat seedlings, a fertilization with NPK fertilizer (12-12-17) was carried out, according to the dosages recommended on the label (50gr / m2).

Pot Test 1

1. "glyphosate-free" water (control);

2. water with glyphosate at a concentration of 0.1 μg / L (corresponding to 0.1 μg / L for the actual duration of the wheat cycle - threshold value of the average annual environmental quality standard);

3. water with glyphosate at a concentration of 1.08 μg / L (corresponding to 1.080 μg / L for the real duration of the wheat cycle - maximum value found in Italian groundwater - 185 monitoring sites - ISPRA 2016 - National pesticide report in the water data 2013-2014);

Pot Test 2

1. "glyphosate-free" water (control);

2. water with glyphosate at a concentration of 100 μg / L;

3. water with glyphosate at a concentration of 1000 μg / L.

For the Pot Tests, the Svevo variety (durum wheat) was used. The cultivation substrate in the pots consisted of soil sampled in Ozzano dell'Emilia (BO), in the fields of the Agricultural University of Bologna. After sampling, the soil was sieved and mixed with river sand, in the following ratio: 80% earth - 20% sand. After an artificial vernalization treatment, the seeds of the Svevo cultivar were sown in pots. Three seeds were sown in each pot. Each treatment involved the

presence of three experimental blocks (replicas) with six pots per block.

The plants under test have reached the stage of full maturation, and therefore it was possible to collect the grain, for both Pot Tests 1 and 2. The samples collected and thus subdivided were sent to Eurofins for the determination of glyphosate and AMPA.

Radiolabeled Experiment

The durum wheat variety (Triticum durum) Svevo was used for the experimental tests. The kernels were germinated on river sand sterilized in Petri dishes kept moist for 48 hours at 4 ° C to stimulate germination. Subsequently the Petri dishes were transferred to a germinator at a temperature of 20 ° C and with a 12 hour photoperiod [50 μmol photons m-2s-1 photosynthetically active radiation (PAR)] for one week. The sprouts were then transferred to plastic vessels (4 cm radius, 350 ml volume, without drainage hole) containing sterilized river sand (approximately 1/3 of the pot volume).

Following what already reported by other authors (Piotrowicz-Cieślak et al. 2018), two different doses of glyphosate have been examined, in a range that guarantees a good percentage of germination and a correct development of the bud, in order to verify the possible uptake and translocation of the molecule.

Two sprouts were placed for each pot. During the experiment, the substrate was daily maintained at the optimum humidity level by adding a ½ Hoagland solution, containing 0.1 mg L-1 glyphosate or 1 mg L-1 glyphosate. In addition, the ½ Hogland solution contained glyphosate- (phosphonomethyl-14C) (Syngenta, Basel, Switzerland) with a radioactivity of 0.5 kBq L-1. At 5, 10 and 20 days from

the beginning of the experimental tests, the plants were weighed, placed in liquid nitrogen, pulverized and extracted with ultrapure water (ratio 1: 4 grams fresh plant weight and ml of water). After centrifugation (15,000 g, 10 min) radioactivity was determined by liquid scintillation spectroscopy (LSS) (1409 Liquid Scintillation Analyzer; Wallac). The experiment was conducted in accordance with a randomized block diagram, with 5 replicates per block. The experiments were repeated twice for sampling times at 5 and 10 days, while for the sampling time at 20 days the experiment was not replicated. Since the effect of the temporal repetition of the experiments was not significant, the different data were combined and subjected to the analysis of variance (ANOVA). Before ANOVA, homogeneity of variance was evaluated with the Bartlett test. Normal and homoskedastic data were analyzed with ANOVA and post-hoc Tukey test. Non-normal and / or heteroskedastic data were analyzed by non-parametric analysis (Kruskall-Wallis test) and post-hoc Dunn's test. The differences were considered significant for P values <0.05. The values reported are expressed as mean ± standard deviation.

3.2 Experimental Safety Studies on in vitro models

Cell cultures

L929 mouse fibroblasts (ATCC-CCL1) were cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco) added with 10 %, fetal bovine serum (FBS, Gibco), 1mM L-glutamine (Gibco) and 1 % penicillin-streptomycin (Gibco).

Caco2 human epithelial cell line (ATCC HTB-37) obtained from colorectal adenocarcinoma were cultured with DMEM added with 10 %, of FBS and 1 % penicillin-streptomycin. Cells were grown in a monolayer condition at 37°C in an atmosphere of 5% CO2 and for the cell treatments, cells were cultured with DMEM alone.

Cell treatments

Glycine (Biosolve), Glyphosate (Sigma-Aldrich) and Roundup®Bioflow were added to cells for 24 hours. Glycine and Glyphosate were diluted in water and the treatments were prepared at different concentrations according the following scheme:

*175 mg/kg * 70 kg (the weight of a common person) / 2 (Lt of daily water).*

The final concentration of the treatments was of 6.125 g/L; 0.6125 g/L ; 0.06125 g/L and 0.175mg/kg and 0.006125 g/L. Each dilution was prepared in DMEM and the pH of each final solution was corrected to 7.0 by adding $NaHCO_3$ (Gibco).

Measurement of cell cytotoxicity for adherent cells

Cell cytotoxicity was measured using the MTT assay. L929 cells (5*104 cells/well) and Caco2 cells (10_5 cells/well) were plated in 96-well tissue culture plate in complete medium (100 µL/well). The multiwell plates were incubated at 37°C, 5% CO_2 for 24 h. After 24 h, the culture medium was removed from the wells and equal volumes (100 µL) of the treatments were added into each well. In control wells, 100 µL DMEM was added. The control wells consisted of untreated cell cultures. 24 hours later, proliferative cells were detected by 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) assay, according to the ISO 10993-

5 International Standard procedure (ISO 10993-5, 2009). The main purpose of the ISO 10993-5 procedure is to define a scheme for testing *in vitro* cytotoxicity of different extracts according to a multi- step approach. Briefly, cells were incubated with MTT solution (1mg/mL, Life Technologies) at 37°C for 2 h. Then, MTT solution was removed and cells were solubilized with 100 µl of isopropanol. The formazan dye formation was evaluated by scanning multiwell spectrophotometer at 540 nm. The results were expressed as viability percentage respect of control.

Statistical analysis

The MTT cell tests were carried out with six replicates for each treatment and data were expressed as mean values of three different experiments. Statistical analysis was performed with R software (R Core Team., 2016). Normal and homoscedastic data were analyzed with ANOVA and Tukey post-hoc tests with Bonferroni correction. Non-normal homoscedastic data were analyzed with the nonparametric Kruskall–Wallis test and Dunn's post-hoc test with Bonferroni correction. Differences were considered to be significant at a p value < 0.05. In the graphs mean values followed by different letters are statistically different.

3.3 Experimental Safety Studies on in vivo models

- **Experimental model**

The study was conducted following the rules established by the Italian law regulating the use and humane treatment of animals for scientific purposes [Decreto Legislativo (D.Lgs.) N. 26, 2014. Attuazione della direttiva n. 2010/63/UE in materia di protezione degli animali utilizzati a fini scientifici. -

G.U. Serie Generale, n. 61 del 14 Marzo 2014]. Before starting, the protocol was examined by the Internal Ethical Committee for approval. The protocol of the experiment was also approved and formally authorized by the *ad hoc* commission of the Italian Ministry of Health (ministerial approval n. 710/2015-PR). The experiment was performed on both male and female SD rats, which belong to the colony used at the Cesare Maltoni Cancer Research Center laboratories of the Ramazzini Institute (CMCRC/RI) for over 40 years. An animal disease screening program enforced by the Italian Health Authority and Research Organization for Animal Health is in place and ongoing on sentinel animals belonging to the RI colony.

Female breeders SD rats were placed individually in Polycarbonate cage (42x26x18cm; Tecniplast Buguggiate, Varese, Italy) with a single unrelated male until evidence of copulation was observed. After mating, matched females were housed separately during gestation and delivery. Newborns were housed with their mothers until weaning. Weaned offspring were housed, by sex and treatment group, not more than 3 per each cage. Cages were identified by a card indicating: study protocol code, experimental and pedigree numbers, dosage group. A shallow layer of white fir wood shavings served as bedding (supplier: Giuseppe Bordignon, Treviso, Italy). Analysis of chemical characteristics (pH, ashes, dry weight, specific weight) and possible contamination (metals, aflatoxin, polychlorobiphenyls, organophosphorus and organochlorine pesticides) of the bedding was performed by CONSULAB Laboratories (Treviso, Italy). The cages were placed on racks, inside a single room prepared for the experiment at 22°C ± 3°C temperature and 50 ± 20% relative humidity. Daily checks on temperature and humidity were performed. The light was artificial and a light/dark cycle of 12

hours was maintained.

During the experiment SD rats received *ad libitum* the standard "Corticella" pellet feed supplied by Laboratorio Dottori Piccioni Srl (Piccioni Laboratory, Milan, Italy). The constituents of the diet are: ground corn (23%), barley milled (15%), soybean meal extract (20.6%), wheat middling (24%), wheat bran (2%), spray dried whey (2.5%), di-calcium phosphate (2%), calcium carbonate (1.1%), chicken meal (6%), carob bean gum (3%), sodium chloride (0.5%), mixed vitamins (0.3%). Every day, the animals drank fresh municipal tap water from glass bottles *ad libitum*. Both feed and water were periodically analyzed to identify possible chemical or microbiological contaminants or impurities; the analyses are included in the documentation of the experiment. The pelleted feed was tested for possible glyphosate contamination in compliance with Commission Regulation (EU) No 293/2013 [maximum residue levels (MRLs) < 1 mg/kg]. Tap drinking water was tested for possible glyphosate contamination in compliance with Directive 2008/105/EC, D.Lgs. 152/2006, Directive2006/118/EC (active substances in pesticides, including their relevant metabolites, degradation and reaction products < 0.1 µg/l).

- **Test Substance**

Active ingredient glyphosate (Pestanal™ analytical standard, CAS number 1071-83-6, purity > 99,5%) was supplied from Sigma-Aldrich (Milan, Italy). The commercial formulation Roundup Bioflow (containing 360 g/L of glyphosate acid in the form of 480 g/l isopropylamine salts of glyphosate (41.5%), water (42.5%) and surfactant (16%; chemical name, CAS number and/or exact percentage have been withheld as a trade secret) was supplied from a local agricultural consortium

(Consorzio Agrario dell'Emilia, Bologna, Italy). The original containers/bottles of glyphosate and Roundup were stored in its original container and kept in a ventilated storage cabinet at room temperature (22°C ± 3°C) throughout the study. Purity data for each batch of glyphosate and Roundup were provided by the supplier. The opening and the use date of the different batches of test substances were recorded in the raw data. An aliquot of each lot of the test article is maintained in the ventilated storage cabinet, until 5 years from the end of the main experiment. The solutions of glyphosate and Roundup were prepared by the addition of appropriate volume of tap drinking water.

- **Experimental plan**

Each of twenty-four virgin female SD rats (17 weeks old, 270-315g) was cohabited outbred with one breeder male rat of the same age and strain. Every day, the females were examined for presence of sperm. Gestational day (GD) 0 was defined as the one in which the sperm was found in vaginal smears. The day on which parturition was completed was designated as lactating day (LD) 0 for the dam and PND 0 for the offspring. Each dam and delivered litter was co-housed in common nesting box during the postpartum period. Following the NTP MOG design, on PND 28, thus 28 days after the last litter was delivered, the offspring were weaned and identified by ear punch according to the Jackson Laboratory system. Sequentially, they were allocated in the same treatment group of their mother in order to have 18 males (8 for the 6-week cohort and 10 for the 13-week cohort) and 18 females (8 for the 6-week cohort and 10 for the 13-week cohort) for each dose group. No more than 2 males and 2 females from the same litter were included in the same cohort/treatment group. Altogether, 108 SD rats (54

males and 54 females) were enrolled in the post-weaning treatment phase. The experimental plan of the pilot study is outlined in Table 1.

Breeders			Offspring				Treatment [b]			End of the experiment	
Group	Animals		Group	Animals [a]							
					Cohort					Cohort	
	Sex	No.	N.	Sex	6-week (No.)	13-week (No.)	Compound	Dose[c]	Age at start [d]	6-week (PND)	13-week (PND)
I	F	8	I	M	8	10	Control (drinking water)	0	GD 6	70[e]	120[f]
	M	8		F	8	10					
	F+M	16		M+F	16	20					
II	F	8	II	M	8	10	Glyphosate	US ADI	GD 6	70[e]	120[f]
	M	8		F	8	10					
	F+M	16		M+F	16	20					
III	F	8	III	F	8	10	Roundup	US ADI Glyphosate equivalent	GD 6	70[e]	120[f]
	M	8		M	8	10					
	F+M	16		F+M	16	20					
TOTAL	M+F	48		M+F	48	60					

[a] No more than 2 sisters and 2 brothers per litter
[b] Test compounds are administered *ad libitum* in drinking water
[c] Doses are calculated considering the Glyphosate US ADI (1.75 mg/kg bw/day)
[d] Solutions are admistered to dams starting from the 6th day of pregnancy
[e] Animals are treated until the landmarks of sexual development are acquired (PND 73 ± 2).
[f] Animals are treated from embryonic life (GD 6) indirectly from dams milk until PND 28 ± 2, then directly for 90 days after weaning (until PND 125 ± 2)

Table 1. *Experimental Plan*

A summary of the endpoints in the pilot study, both in dams and in the offspring (6-week and 13-week cohorts) is presented in Table 2.

Group		Body weight	Water and feed cons.	Urinanalysis	Clinical chemistry	Haematological tests	Organ weight	Histopathology	Micronuclei	Transcriptome	Microbiome	Litter size	Live birth index	Sex ratio	Intra/extra uterine death	Anogenital distance	Balano-preputial sep.	Vaginal opening	First estrous	Estrous cyclicity	Hormone analyses	Sperm analyses	Sperm aneuploidy
I (control)	F0	√	√				√	√	√		√										√		
	F1	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
II (Glyphosate)	F0	√	√				√	√	√		√				√						√		
	F1	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
III (Roundup)	F0	√	√				√	√	√		√				√						√		
	F1	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√

Table 2. *Endpoints of the study evaluated in dams (F0) and offspring (F1)*

Two groups of SD rats were treated with either glyphosate or Roundup diluted in tap water administered *ad libitum* and one group received only tap water as control. Roundup was diluted in tap water in order to obtain an equivalent dose of glyphosate of 1.75 mg/kg bw/day. During gestational and lactational periods, embryos and newborns (F1) received the test compounds mainly through their dams (F0). Glyphosate and Roundup water formulations during these periods were freshly prepared on a daily base depending on individual body weight and water consumption of dams as measured at each scheduled time point (see below). After weaning, until the end of the experiment (PND 73 ± 2 or 125 ± 2), the test substances were administered in tap water to F1 animals on the basis of the average body weight and average water consumption *per* sex and *per* experimental group, as measured at each scheduled time point (see below). Males and females were considered separately because of their difference in weight gain, body weight and water consumption.

At least every week, the exposure doses were recalculated and registered. The actual levels of test compounds that reached the fetus during gestation or that were ingested postnatally by the offspring during the period of lactation were not estimated in the present study.

Animals were monitored during the entire experimental period. The following procedures were performed:

- Health status control: from the start of the experiment, animals were checked three times daily, except on Sundays and non-working days, when they were only checked twice. All observed variations from normal status were recorded.
- Clinical control: status, behavior and clinical observation on the experimental

animals were checked before the start of the treatment, and at least every two days until the end of the experiment. Any findings listed below were then recorded: alterations of skin, hair, eyes and mucosa; modification in production of secretions or excretions and in autonomic activity; respiratory symptoms; postural changes or changes in walk; presence of tonic or clonic contractions; unusual stereotypes and behavior.

Dams' body weights were recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, dams' body weights were recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Pups' body weight by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. After weaning, the body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifices; the means of individual body weights were calculated for each group and sex.

Dams' feed and water consumption were recorded twice weekly during gestation (GD 0, 3, 6, 9, 12, 15, 18, 21), whereas during lactation were measured at LD 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28.

After weaning the daily water and feed consumption *per* cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2; the means of individual consumptions were calculated for each group and sex.

The day before the terminal sacrifices, all the animals were located individually in metabolic cages and starved for around 16 hours. During this time, the animals had free access to water alone or to the programmed test compound solutions. The day after, in the morning, samples of at least 5ml of spontaneous urine from each animal were collected and put in separate labelled tubes. Urine samples for

analysis of glyphosate and AMPA excretion were obtained from 3 dams/group and from 10 (5 males + 5 females) rats/group belonging to the 6-week and 13-week cohorts.

- Glyphosate and aminomethylphosphonic acid (AMPA) detection

Analyses of glyphosate and its metabolite AMPA in drinking water, feed and urine were performed by Neotron Laboratories (Modena, Italy), who is a laboratory officially accredited by Accredia (Lab. N. 0026) according to European regulation UNI CEI EN ISO/IEC 17025:2005. The specification and results are maintained in the experimental documentation. The analytical method is based on liquid chromatography tandem mass spectrometry (LC-MS/MS) (FDA 2002a, b; Granby et al. 2003; Kubilius and Bushway 1998). The limit of quantification (LQ) for glyphosate and AMPA corresponded to 0.10 µg/l in water, 50 µg/kg in feed, and 1 µg/kg in urine.

4. Bacterial 16S PCR and sequencing

Rat fecal DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total DNA concentration was determined by Qubit 2.0 Fluorometer (Life technologies, Norwalk, CT). The phylogenetically informative V3–V4 region of 16S rRNA gene was amplified using universal primer 347F/803R[33, 34] with dual-barcoding approach previously described[35]. The integrity of the 16S PCR amplicons was verified by agarose gel electrophoresis. The resulting ~460-bp sized amplicons were pooled and then sequenced with the Illumina MiSeq 2x250 paired-end sequencing platform at OCS genome technology center of New York University Langone Medical Center.

5. 16S data analysis

The sequencing data were merged and filtered to remove the merged reads with a length of <400bp or the quality score of < Q30 at more than 1 % of bases. Sequentially, all filtered high quality reads were split by dual-barcode and trimmed of primer regions using a self-defined bash script to integrate several sequencing processing commands from fastx, QIIME, and seqtk. Duplicated measurements of four sample were processed and sequenced using different barcodes to test the sequencing reproducibility. Five blank samples were also sequenced and referenced to filter the possible environmental contamination during the sample procession. The split high-quality reads were further processed by QIIME 1.9.0[37]. We used the command *pick_open_reference_otus.py* with the defaulted green_gene 97_otus reference sequences to cluster of >97% similar sequencing reads as an OTU using uclust[40]. Representative sequences for each OTU were aligned using PyNAST and build the phylogenetic tree.

The diversity within each microbial community, so-called alpha-diversity, was calculated using the Shannon Index[41] as metric and represented the measure of the diversity at the family and genus level. The overall microbiome dissimilarities among all samples were accessed using the weighted UniFrac distance matrices [42]. Non-metric multiple dimensional scaling (NMDS) were used to visualize the dissimilarities. The permutational multivariate analysis of variance PERMANOVA test [43], with the maximum number of permutations = 999, was performed to assess the significance of the overall microbiome differences between groups by collection timepoints and treatment. The PERMANOVA procedure using the [Adonis] function of the *R* package *vegan* 2.0–5 [44]

partitions the distance matrix among sources of variation, fits linear models to distance matrices and uses a permutation test with pseudo-*F* ratios to obtain the *p* values. Using the LEfSe method[45], we further selected the microbiome features significantly associated to time of collection and treatments at various taxonomic ranks.

- **Statistical analysis**

Summary statistics, means ± standard deviations (Zauber et al.), were calculated for continuous variables. For body weight, water and feed consumption over time further analyses were performed using multilevel mixed-effect linear regression models, to control for within subject correlation across time; moreover we have considered also the litter effect during the lactation period. Analysis of variance and Dunnett's tests (when applicable) were also performed to compare body weight gain in different periods and consumption of food and water as mean consumption in several periods.

All tests were two tailed, with alpha set at 0.05. Statistical analyses were perfomed by using STATA version10 (Stata Corporation, College StationTexas, USA).

4. RESULTS AND DISCUSSION

4.1 Experimental Absorption Studies on Wheat Plants

Pot Test 1

Overall, 54 plants were grown per group, divided into three replicas of 18 plants each. The trial lasted 103 days, and the volume of treatment administered was

60 L, or about 1.1 L per plant. The results of Pot Test 1 are reported in Table 3.

	Roots + collar	**Stem**	**Grain**
Group 1 (untreated control)	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb
Group 2 (0.1 μg / L glyphosate)	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb
Group 3 (1.08 μg / L glyphosate)	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb

Table 3. *Pot Test 1: Evaluation of AMPA and Glyphosate residues in plants exposed at different concentrations (0.1 μg / L; 1.08 μg / L) of glyphosate in water*

The grain samples related to Group 1, 2, 3 tested all negative, ie with glyphosate values below the LOD. Furthermore the glyphosate and AMPA values were below the LOD also for stem and root + collar samples in all three groups under examination.

Pot Test 2

Overall, 54 plants were grown per thesis, divided into three replicas of 18 plants each.The trial lasted 105 days, and the volume of treatment administered was 90 L, or about 1.7 L per plant. The duration of the cycle was overall equal to what happened during the first trial (103 days vs 105 days), but the volume of water administered during the test was increased. this might be attributable to the fact that, in this last test, the external temperatures were much higher than the first test and therefore the greater the evaporation-transpired water. The results of Pot Test 2 are reported in Table 4.

	Roots + collar	**Stem**	**Grain**
Group 1 (untreated control)	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb
Group 2 (100 µg / L glyphosate)	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb
Group 3 (1000 µg / L glyphosate)	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb	Glyphosate: <0.01 ppb AMPA: <0.01 ppb

Table 4. *Pot Test 2: Evaluation of AMPA and Glyphosate residues in plants*

exposed at different concentrations (100 µg / L; 1000 µg / L) of glyphosate in water

The grain samples related to Group 1, 2, 3 tested all negative, ie with glyphosate values below the LOD. Furthermore the glyphosate and AMPA values were below the LOD also for stem and root + collar samples in all three groups under examination.

Radiolabeled Experiment

In the test carried out by treating the plants with the lowest concentration of glyphosate (0.1 mg glyphosate L-1), no absorption and / or translocation of the radiolabelled molecule was detected in any of the times examined. With regard to the test performed with the highest concentration of glyphosate (1 mg glyphosate L-1), significant differences were observed both as a function of the treatment and as a function of the time taken. The average dry weight of the treated and control samples was found to vary significantly depending on the time, but not according to the treatment (Figure 1). This evidence shows that the treatment dose used did not interfere with the normal growth of the plant. Furthermore, we note an excellent development up to 10 days after the treatment, where a daily growth rate of about 0.37 g is detected. Between 10 and 20 days, the observed dry weight value appears to vary significantly, but the growth rate is significantly slowed (0.033 g per day).

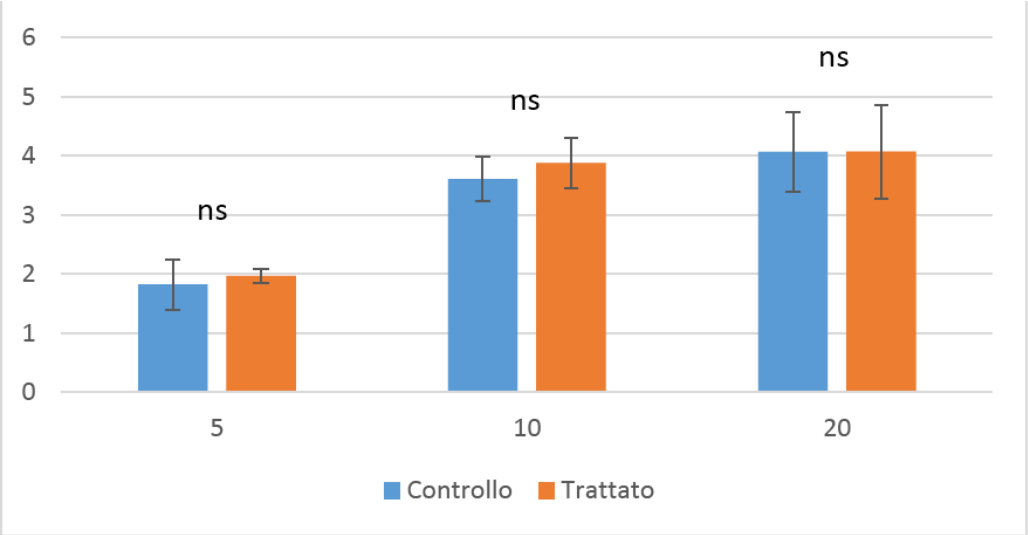

Figure 1. *The histograms show the average dry weight measured in the control samples (in blue) and in the treated samples (in orange). The data refer to 5, 10 and 20 days after the treatment*

As for the study of the absorption of radiolabeled glyphosate, the test was carried out using an inert substrate (sand) sterilized, in order to eliminate the possible presence of microorganisms. Therefore it is reasonable to assume that all the glyphosate present in the growth substrate is in an "available" form for absorption by wheat roots. Despite this, the proportion of glyphosate absorbed via the root was very low and always below 5%.

As reported in Figure 2, the proportion of glyphosate absorbed (expressed as a percentage of the applied glyphosate) varies significantly after 5 days from the treatment compared to 10 and 20 days after the treatment. However, no significant difference is found between the proportion of glyphosate absorbed at

10 and 20 days after treatment. In particular, at 5 days after treatment, 1.97% of the applied glyphosate is absorbed, while at 10 and 20 days after treatment the proportion of glyphosate absorbed rises to 3.88% and 4.08% respectively. From the analysis of the obtained data there is a weak but significant correlation ($r = 0.38$; $p < 0.01$) between the share of glyphosate absorbed and the dry weight of the plant, showing a higher accumulation of glyphosate in larger plants. Regarding the translocation of the glyphosate absorbed through the roots, a substantial balance was observed between the share divided in the root and epigeal portion of the shoots: at 20 days from the treatment 54 ± 5% of the total radioactivity in the seminal radicles and the remaining 46 ± 2% in green fabrics. Similar values were observed at 5 and 10 days after treatment.

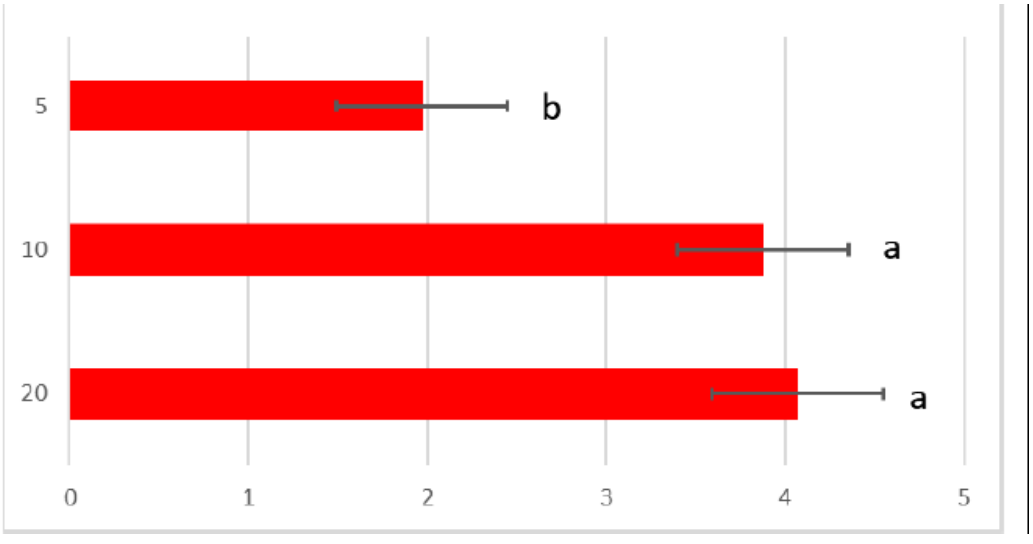

Figure 2. *The histograms show the percentage of 14C-glyphosate absorbed and detected in plants. The data refer to 5, 10 and 20 days after the treatment.*

On the basis of the experimental data it is possible, with a good approximation, to exclude that glyphosate concentrations comprised between 0.1 and 1.08 ppb (μg / L), present in the circulating solution of a clayey soil (type of very common soil, if not prevalent in most of the agricultural area of the Po Valley), assuming a stratum at a depth of 20 cm, it can induce detectable contamination in the tissues and in the grain of durum wheat. It is important to underline that the tests conducted, reasonably, allow to exclude possible contamination of the grain of durum wheat following phenomena of translocation of glyphosate, possibly present in groundwater, in a soil not contaminated by the active ingredient, but obviously does not exclude other forms of possible contamination not subject to investigation in the present project (for example due to direct incorporation of the herbicide in the ground).

Using the average temperatures collected in our areas (Bologna) and assuming a crop cycle for durum wheat of about 250 days (with sowing carried out on 14/10 and harvesting carried out on 20/06), the calculation of potential evapotranspiration according to the Hargreaves-Samani formula it determines a value equal to 422 mm (Figure 3). From this value it follows that, considering an average production per ha of 5 t, there will be a water consumption of 844.2 L / kg of grain produced, or equal to 1.7 L per ear. At the concentrations of glyphosate taken into consideration in the Pot Tests (0.1 and 1.080 μg / L) and assuming that all the water absorbed is translocated in the grain, at the end of the crop cycle, a concentration of glyphosate can be expected of 85 and 910 respectively ppb.

Assuming, on the other hand, a partial translocation in the grain (equal to 25%) in the initial phases of the plant's biological cycle, that is from sowing to raising, and a translocation equal to 75% of the active principle absorbed by the plant from the phase of the barrels up to the vitreous maturation, the simulation indicates a minimum contamination equal to 30-40 ppb, in the case of exposure to groundwater with a concentration equal to 0.1 µg / L, and equal to 400-420 ppb, in the case of exposure to groundwater with concentration equal to 1.08 µg / L. Overall, the simulations suggest that we might expect contamination values compatible with what is currently detected in semolina or in processed products, marketed in Italy. Moreover, these concentrations are above the instrumental LOD and therefore they would in theory detectable in our Pot Experiments.

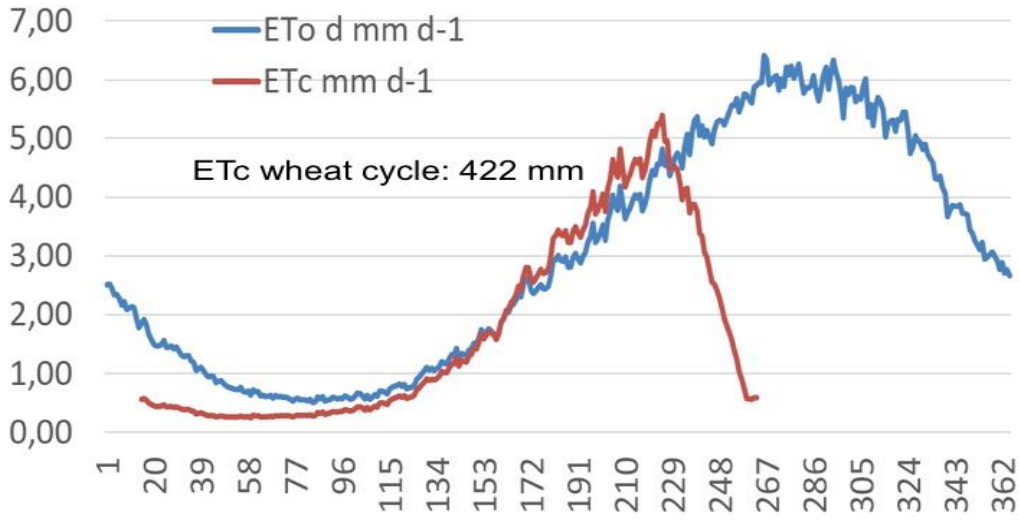

Figure 3. *Simulation of the water consumption of wheat in the Po Valley. The weather data refer to the temperature and precipitation averages for the 1998-2017 period. The red line indicates the real evapotranspiration of the wheat simulated in function of the potential evapotranspiration calculated on the basis of historical weather data (blue line).*

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Although the predicted concentrations of the model are well above the instrumental LOD, the present tests carried out in a controlled environment do did not confirm what was hypothesized. The prediction was based on the assumption that the active principle is passively absorbed at a radical level by xylematic flow: the present experimental tests suggest that the absorption of glyphosate is not passive. In order to estimate the percentage of active principle absorbed by the circulating solution, two experimental tests were carried out with radiolabeled glyphosate, using optimal experimental conditions for the absorption of the active principle by radical route (shoots on sandy substrate soaked with solutions of glyphosate at a concentration in the order of mg / L), according to the available literature. In the test conducted at a concentration of 0.1 mg / L, no absorption and / or translocation of the radiolabelled molecule was detected in any of the times examined. As regards the test performed with the highest concentration of glyphosate (1 mg / L), significant differences were observed both as a function of the treatment and as a function of the relief time: the maximum absorption was observed at 20 days from the treatment with values of around 4%. Glyphosate, once absorbed, is translocated apically, and divided almost equally between the root system and the epigeal portion of the young plant. The data obtained are completely similar to those observed for soy sprouts, placed in experimental conditions similar to those of the present study (Piotrowicz-Cieślak et al. 2018).

Under optimal conditions, the radical absorption of glyphosate by young durum wheat plants is in the order of 4% and is comparable to that observed in other

species of agricultural interest. Absorption appears to be dependent on the concentration of active ingredient.

4.2 Experimental Safety Studies on in vitro models

In order to study the effect of Glycine, Glyphosate and Roundup® on cell proliferation, mouse fibroblasts L929 and human intestinal cells Caco2 were plated in 96 well plates, 6 wells for each condition and treated for 24 hours with 4 doses of the three treatments (0,175, 1,75, 17,5 and 175 mg/kg). MTT assay was performed 24 hours later and each experiment was repeated three times. Statistical analysis one way Anova was performed and different letters indicate statistically different mean values at $P<0.05$. Glycine didn't modulate nor L929 or Caco2 cell proliferation at none of the doses used for cell treatments. Glyphosate showed a positive correlation between the percentage of proliferating cells as a function of the concentration for both L929 cells ($R=0.957$, Figure 4) and Caco2 cells ($R=0.986$, Figure 4). For both cell types, it reduced cell proliferation in a significative way only at the highest dose of 175 mg/kg. Roundup® showed a positive correlation between the percentage of proliferating cells as a function of the pesticide concentration for both L929 and Caco2 cells, with $R=0.956$ and $R=0.978$ respectively (Figure 4). It showed significative effects for the doses of 17.5 and 175 mg/kg compared to the untreated control.

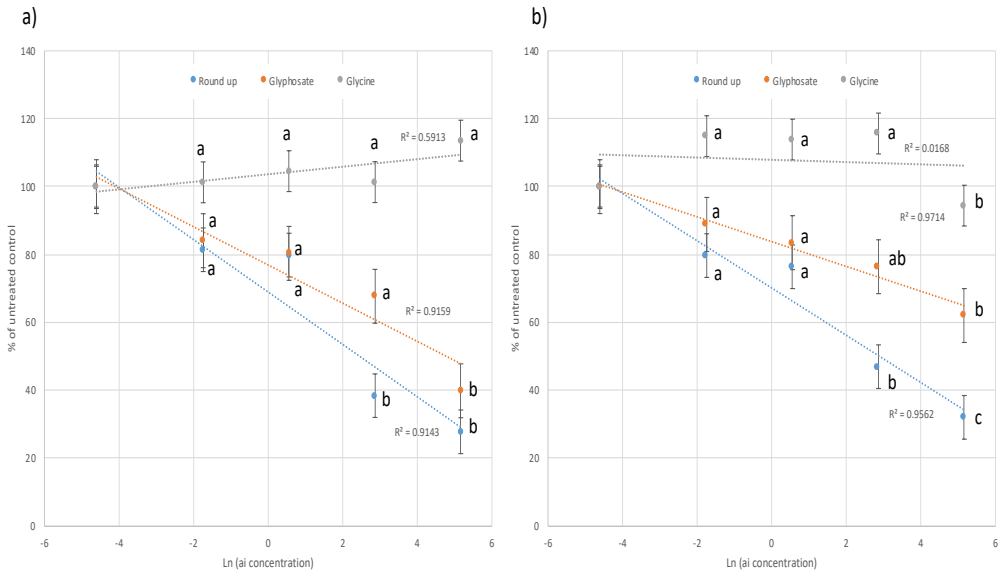

Figure 4 *MTT test on L929 (a) and on Caco2 (b). Three independent experiments, 4 doses (0.175, 1.75, 17.5, 175 mg/kg), 6 replicates. One way Anova (different letters indicates statistically different mean values at P<0.05)*

The MTT results seem to indicate a different mechanism of action of glycine and its analogue glyphosate (and its formulation Roundup). Our results confirm previous evidence of cytotoxicity of glyphosate in in vitro models on human hematological cell lines (Raji) (Townsend et al. 2017). Our findings seem to support the hypothesis of a higher cytotoxic potency of the formulation, compared to pure glyphosate, in line with what observed by other authors (Wozniak et al. 2018) and with the results of the in vivo studies.

4.3 Experimental Safety Studies on in vivo models

BIOACCUMULATION

The results of glyphosate and AMPA urinary concentrations are reported in Table 5 and Figure 5.

		Dams		Offspring (6-week cohort)		Offspring (13-week cohort)	
	Treatment	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)
Male	Control			0.012 ± 0.010	0.003 ± 0.003	0.011 ± 0.010	0.006 ± 0.004
	Glyphosate	–	–	0.938 ± 0.414	0.014 ± 0.007	1.684 ± 0.768	0.023 ± 0.012
	Roundup			1.174 ± 0.439	0.011 ± 0.005	2.280 ± 1.520	0.027 ± 0.016
Female	Control	0.009 ± 0.001	0.006 ± 0.002	0.013 ± 0.007	0.005 ± 0.001	0.008 ± 0.005	0.003 ± 0.005
	Glyphosate	0.480 ± 0.010	0.024 ± 0.002	0.938 ± 0.377	0.016 ± 0.010	1.354 ± 0.359	0.013 ± 0.006
	Roundup	0.700 ± 0.106	0.024 ± 0.001	0.910 ± 0.383	0.018 ± 0.007	1.524 ± 0.585	0.021 ± 0.007

Table 5. *Glyphosate and AMPA concentration in urine. Results are reported as mean ± standard deviations*

The urinary concentration of both glyphosate and AMPA of SD rats treated with 1.75 mg/kg bw/day of glyphosate were comparable to the ones observed in SD rats treated with Roundup dose equivalent to 1.75 mg/kg bw/day, despite limited sample size and the large standard deviations. In the control group, as expected, the glyphosate and AMPA urinary levels were all below or close to the limit of quantitation (0.001 mg/kg).

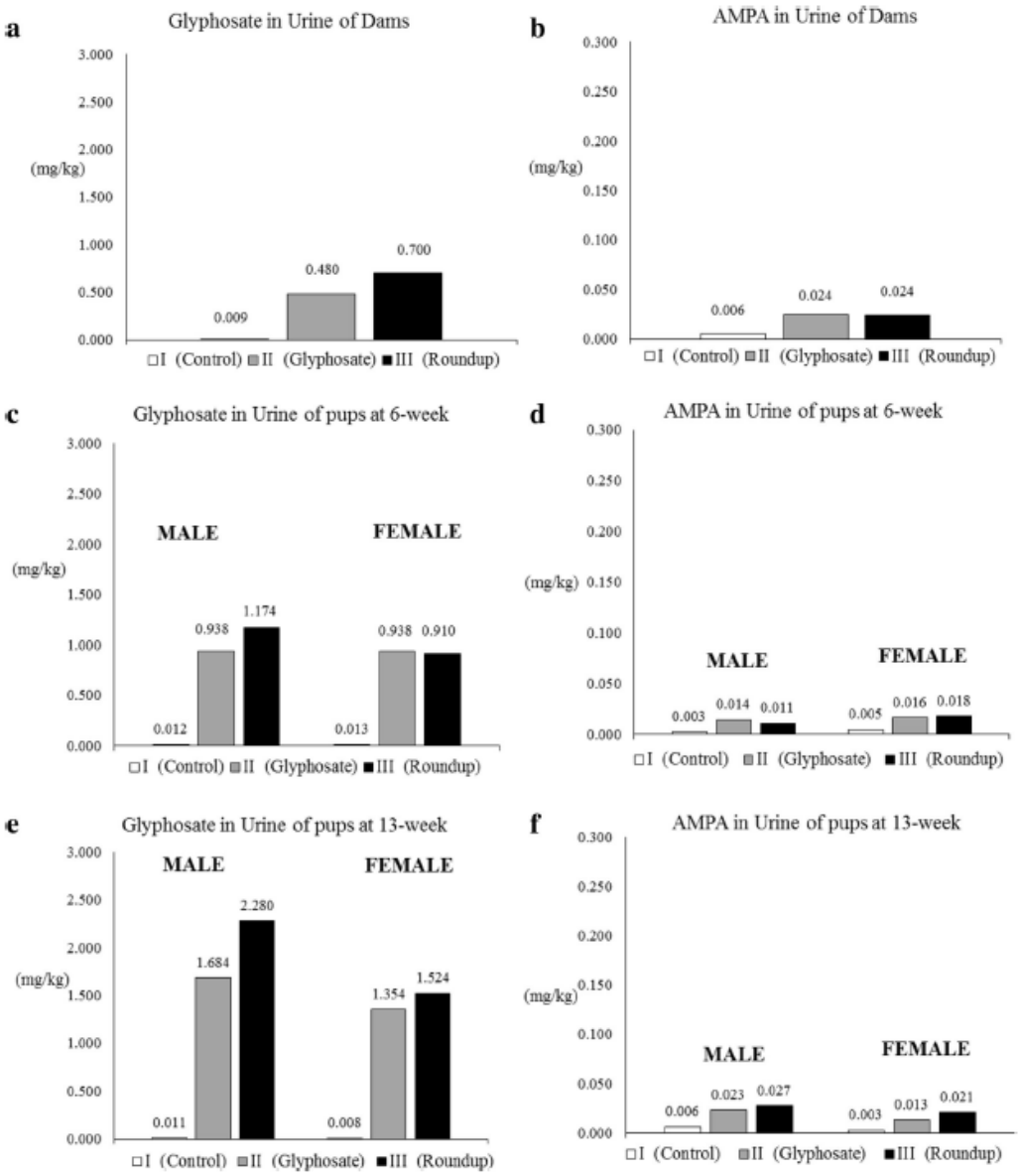

Figure 5. *Average urinary concentrations of glyphosate and AMPA, expressed in mg/kg, collected at terminal sacrifices. Dams glyphosate (a) and*

AMPA (b) excretion; 6-week cohort male and female offspring; glyphosate (c) and AMPA (d) excretion; 13-week cohort male and female pups

Glyphosate (e) and AMPA (f) excretion

In the treated SD rats, the majority of glyphosate was excreted unchanged (as parent compound), with urinary levels about 100-fold higher than that of its metabolite AMPA. For example, glyphosate and Roundup treated females in the 13-week cohort presented mean urinary levels of glyphosate respectively of 1.354 mg/kg and 1.524 mg/kg, while the AMPA levels were respectively 0.013 mg/kg and 0.021 mg/kg. In glyphosate and Roundup treated SD rats, a time-dependent increase in the mean urinary concentration of glyphosate was observed. In glyphosate and Roundup treated males, an approximate 2-fold increase in the 13-week cohort (animals exposed prenatally until 125±2 days after birth) compared to the 6-week cohort (animals exposed prenatally until 73±2 days after birth) was observed. In glyphosate treated females, the 6-week cohort (animals exposed prenatally until 73±2 days after birth) showed a 2-fold higher value than the dams after weaning (exposed for 49±2 days), while the 13-week cohort (animals exposed prenatally and 125±2 days after birth) showed a 1.5-fold increase compared to the 6-week cohort. In the Roundup treatment group, the increase was less steep, but the time-dependent pattern was still evident. In glyphosate and Roundup treated SD rats, the levels of AMPA were comparable at the different time points in both males and females. In these animals, large standard deviations of the values of AMPA concentrations in urine have been observed, in particular for values close to the limit of quantitation as in the control groups.

Both glyphosate and Roundup exposure led to comparable urinary concentrations of glyphosate and AMPA with an increasing pattern of glyphosate excreted in urine in relation to the duration of treatment, indicating the systemic bioavailability of the active substance. The adjuvants and the other substances present in Roundup did not seem to exert a major effect on the absorption and

excretion of glyphosate.

MICROBIOME

Microbiome profiling revealed that low-dose exposure to Roundup® and glyphosate resulted in significant and distinctive changes in overall bacterial composition in F1 pups only. Specifically, at PND31, corresponding to pre-pubertal age in humans, relative abundance for *Bacteriodetes* (*Prevotella*) was increased while the *Firmicutes (Lactobacillus)* was reduced in both Roundup(x) and glyphosate exposed F1 pups compared to controls. Furthermore, our results showed that the overall microbiome diversity and composition were significantly different between Roundup and glyphosate, suggesting possible synergistic effects of the mixed formulation on gut microbiota. As most of GBHs contains multiple surfactants and adjuvants might act differently than glyphosate alone, it is not only important to understand the individual effects of glyphosate, but also the synergistic impact of mixed formulations. In fact adjuvants might act alone or in a synergistic manner and increase the toxic effects of glyphosate.

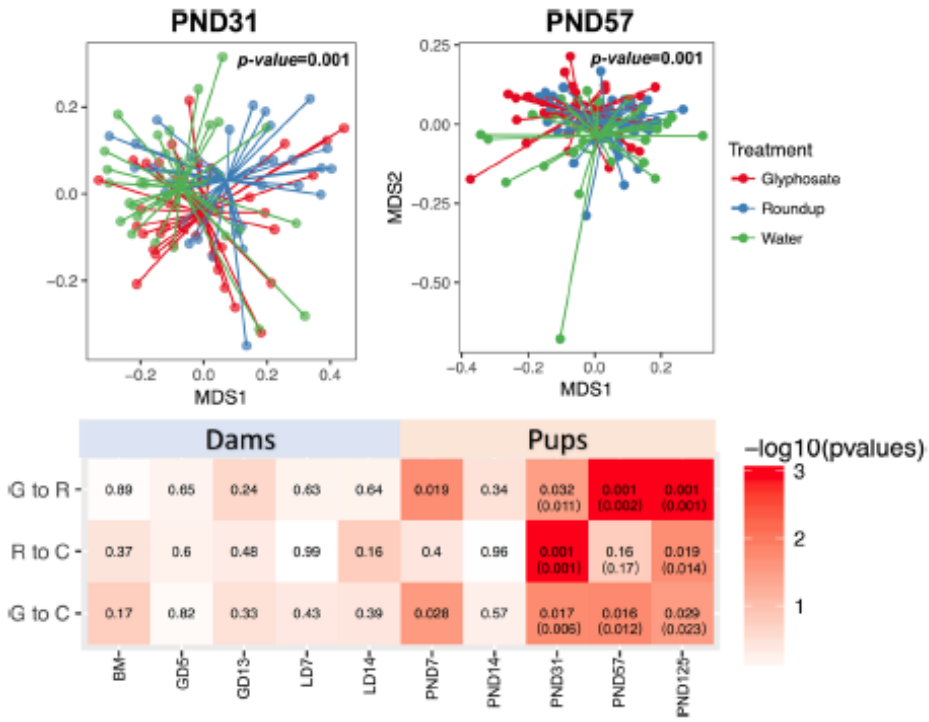

Figure 6. *The effect of glyphosate exposure on overall microbiome diversity. a NMDS plots visualize the overall microbiome dissimilarities (beta-diversity) between individual rat of three treatments at PND 31 and PND 57. b PERMANOVA test is performed to test the significance among all three treatments (displayed in NMDS plots) and between two treatments (values are listed in tables). The p-values in parenthesis were adjusted for genders. G: glyphosate; R: Roundup; C: control water*

REPRODUCTIVE DEVELOPMENTAL

AGD at PND4 was statistically significantly increased both in Roundup-treated males ($p < 0.01$) and females ($p < 0.01$) and in glyphosate-treated males ($p < 0.01$) [multilevel linear regression considering individual pup body weight] (Table 6). Even applying a multilevel linear regression model with litter as random effect, the AGD still resulted to be increased in a statistically significant manner in both

Roundup-treated males ($p < 0.01$) and females ($p < 0.01$) Post weaning body weights as well as water and feed consumption were homogenous across exposure groups in both female and male offspring (Table 6).

Parameter	Control	Glyphosate	Roundup
Number of male pups at PND 1	58	46	53
Male pups weight at PND 1 (g)[a]	6.8 ± 0.5	7.1 ± 0.2	6.8 ± 0.4
Male pups weaning weight (g)[a, b]	50.4 ± 4.4	53.5 ± 6.0	51.8 ± 5.8
Male AGD (mm) at PND 4[a, c]	4.02 ± 0.49	4.26 ± 0.38**	4.34 ± 0.30****
Age (PND) at balano-preputial separation (BPS)	46.33 ± 1.85	46.78 ± 1.73	47.61 ± 2.77
Body weight at BPS (g)	202.50 ± 10.74	203.89 ± 16.68	207.50 ± 22.70
Number of female pups at PND 1	51	61	60
Female pups birth weight (g)[a]	6.4 ± 0.4	6.6 ± 0.4	6.5 ± 0.6
Female pups weaning weight (g)[a, b]	48.3 ± 5.1	50.4 ± 5.2	50.5 ± 5.1
Female AGD (mm) at PND 4[a, c]	1.70 ± 0.25	1.79 ± 0.21	1.86 ± 0.19****
Age (PND) at vaginal opening (VO)[a]	35.56 ± 1.72	35.39 ± 1.5	35.61 ± 1.14
Body weight at VO (g)[a]	108.33 ± 6.18	108.06 ± 7.10	109.44 ± 8.73
Age (PND) at First Estrous (FE)[a, d]	39.88 ± 1.25	40.13 ± 1.46	42.63 ± 3.25#
Number of days between VO and FE[a]	4.75 ± 0.71	5.13 ± 0.64	7.00 ± 3.78

**Statistically significant ($p < 0.01$) with multilevel linear regression adjusted for body weight
*Statistically significant ($p < 0.01$) with multilevel linear regression adjusted for body weight and litter (random effect)
#Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
[a]Mean ± standard deviation
[b]Weaning weight corresponds to PND 25
[c]AGD = ano-genital distance
[d]First estrous (FE) was evaluated only in females belonging to the 6 week cohort

Table 6. *Effects of glyphosate or Roundup Bioflow exposure on developmental landmarks and sexual characteristics of pups*

in female offspring age and body weight at VO was similar across treatment groups; however, age at FE was significantly delayed ($p < 0.05$) with Roundup treatment (Table 6). All the females offspring of the control- and glyphosate-treated groups presented the FE within 6 days from the VO, while in the Roundup-treated group two out of ten females presented a more than doubled interval between VO and FE (one after 12 days and the other after 14 days). TT serum levels significantly increased in female offspring rats treated with Roundup from the 13-week cohort compared to control animals ($p < 0.05$); TT showed a numerical, but not statistical, increase also in the Glyphosate group. No significant differences in serum levels of TT were observed in younger female offspring from the 6-week cohort. Both male and female Roundup-treated animals

belonging to the 13-week cohort showed a marked decrease in DHT/TT ratio ($p < 0.01$). No statistically significant differences were observed in younger males and females (6-week cohort).

GBHs exposure was associated with androgen-like effects, in particular in females, including a statistically significant increase of AGDs in both males and females, delay of FE and increased testosterone in females. GBHs exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13-week)

5. CONCLUSIONS

Considering that all experimental absorption studies on wheat plants have given negative results in terms of the presence of glyphosate or AMPA in the grain of durum wheat, the main conclusions that can be drawn are the following:

1- the phenomenon of "involuntary" contamination of the grains of durum wheat from glyphosate and / or AMPA, caused by the presence of glyphosates in the groundwater or in the circulating soil solution, is not demonstrable up to the concentration of 1 mg / L of glyphosate in groundwater (concentrations of glyphosate and AMPA <LOD in wheat roots, collar, stem and grain);

2- The tests carried out cannot exclude that other forms of grain contamination might occur (not specifically tested in the present research project), such as the possible translocation of glyphosate, incorporated directly into the soil or trough drift effects (aerosol), due to the effect of any total weeding treatments repeated

in the unit culture;

The findings from the experimental safety studies on in vitro and in vivo models highlighted different effects at doses currently considered safe for humans and with no effects in animals. In particular:

- Cytotoxic effects were observed in MTT in vitro assays in murine (L929) and human (Caco) cells at treated with GBHs at doses that were considered with no effects in animals (17.5, 175 mg/kg). The formulation Roundup Bioflow seem to be more toxic than pure glyphosate.
- In in vivo models Exposure to GBHs at doses considered safe in humans (1.75 mg/kg bw/day, equivalent to the US ADI) altered the gut microbiota of rats in early development, particularly before the onset of puberty. Exposure to GBHs was also associated with androgen-like effects, including a statistically significant increase of anogenital distance (AGD) in males and females, delay of first estrous and increased testosterone in females. Furthermore, a statistically significant increase was observed in micronuclei in rats treated with GBHs, especially in the first part of life. The concentration of both glyphosate and AMPA in the urine of SD rats treated with glyphosate was comparable to that observed in animals treated with Roundup, with an increase related to the duration of treatment.

Overall the integration of these findings from absorption in plants and safety studies will serve as solid evidence-base for risk assessment and productive strategies for agriculture.

6. THESIS RELATED ACTIVITIES AND PUBLICATIONS

The candidate followed the Industrial PhD Programme in Agricultural, Environmental, and Food Science and Technology (STAAA) of the Department of Agricultural and Food Sciences (DISTAL, University of Bologna) under the supervision of Prof. Giovanni Dinelli (DISTAL, University of Bologna) and Dr. Fiorella Belpoggi (Ramazzini Institute). The activities and work performed during the PhD programme led to the publication of three manuscripts in international peer-reviewed journals:

- *Panzacchi S*, Mandrioli D*, Manservisi F, Bua L, Falcioni L, Spinaci M, Galeati G, Dinelli G, Miglio R, Mantovani A, Lorenzetti S. The Ramazzini institute 13-week study on glyphosate-based herbicides at human-equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation. Environmental Health. 2018 Dec;17(1):52.*

- *Manservisi F*, Lesseur C*, Panzacchi S, Mandrioli D, Falcioni L, Bua L, Manservigi M, Spinaci M, Galeati G, Mantovani A, Lorenzetti S, Miglio R, Andrade AM, Kristensen DM, Perry MJ, Swan SH, Chen J, Belpoggi F. The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human equivalent dose to Sprague-Dawley rats: effects on development and endocrine system. Environmental Health. 2019 Dec;18(1):15.*

- *Qixing Mao*, Fabiana Manservisi*, Simona Panzacchi, Daniele Mandrioli, Ilaria Menghetti, Andrea Vornoli, Luciano C Bua, Laura Falcioni, Corina Lesseur, Jia Chen, Fiorella Belpoggi, Jianzhong Hu. The Ramazzini Institute 13-Week Pilot Study On Glyphosate And Roundup Administered At Human-Equivalent Dose To Sprague Dawley Rats: Effects On The Microbiome. Environmental Health. 2018 Dec;17(1):50.*

Some of the findings described in this PhD work have been presented before the EU Parliament on May 16th 2018.

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