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Environmental and Toxicological Impacts of Glyphosate with Its Formulating Adjuvant

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***Abstract*—**Environmental and toxicological characteristics of formulated pesticides may substantially differ from those of their active ingredients or other components alone. This phenomenon is demonstrated in the case of the herbicide active ingredient glyphosate. Due to its extensive application, this active ingredient was found in surface and ground water samples collected in Békés County, Hungary, in the concentration range of 0.54–0.98 ng/ml. The occurrence of glyphosate appeared to be somewhat higher at areas under intensive agriculture, industrial activities and public road services, but the compound was detected at areas under organic (ecological) farming or natural grasslands, indicating environmental mobility. Increased toxicity of the formulated herbicide product Roundup compared to that of glyphosate was observed on the indicator aquatic organism *Daphnia magna* Straus. Acute LC_{50} values of Roundup and its formulating adjuvant polyethoxylated tallowamine (POEA) exceeded 20 and 3.1 μg/ml, respectively, while that of glyphosate (as isopropyl salt) was found to be substantially lower (690-900 μg/ml) showing good agreement with literature data. Cytotoxicity of Roundup, POEA and glyphosate has been determined on the neuroectodermal cell line, NE-4C measured both by cell viability test and holographic microscopy. Acute toxicity (LC_{50}) of Roundup, POEA and glyphosate on NE-4C cells was found to be 0.013±0.002%, 0.017±0.009% and 6.46±2.25%, respectively (in equivalents of diluted Roundup solution), corresponding to 0.022±0.003 and 53.1±18.5 mg/ml for POEA and glyphosate, respectively, indicating no statistical difference between Roundup and POEA and 2.5 orders of magnitude difference between these and glyphosate. The same order of cellular toxicity seen in average cell area has been indicated under quantitative cell visualization. The results indicate that toxicity of the formulated herbicide is caused by the formulating agent, but in some parameters toxicological synergy occurs between POEA and glyphosate.

***Keywords*—**Glyphosate, polyethoxylated tallowamine, Roundup, combined aquatic and cellular toxicity, synergy.

I. Introduction

PESTICIDES are applied in agriculture in form of formulated preparations, and although only their active ingredients are supposed to exert the desired biological effects, their activity is often modulated by numerous substances that are being used as formulating agents or additives. Formulating agents (detergents, adjuvants, solubilizers, etc.) are supposed to be biologically inert substances; their role is solely to advantageously modify stability, uptake or other physico-chemical properties of the active ingredient in the formulated form.

Glyphosate (N-(phosphonomethyl)glycine) is currently the most notified and best-selling herbicide active ingredient in the world and its market continues to grow in line with the increase in the cultivation of glyphosate-tolerant (GT) transgenic crops [1]. Due to their overall phytotoxicity glyphosate-based herbicides are traditionally used in pre-emergent applications to vegetation control of almost all weeds, but post-emergent use have also become possible on cultivated GT crops. Glyphosate blocks the biosynthesis of essential aromatic amino acids by inhibiting the shikimic acid metabolic pathway existing in plants, fungi and bacteria [2], [3]. Due to its high solubility in water (11.6 g/l at 25°C) and its extensive agricultural usage, glyphosate have been indicated to have the potential to spread in the ecosystem and reach unintended plants, animals and the food chain. It may contaminate surface or ground waters and the exposure of non-target aquatic organisms is a concern in ecotoxicology. In turn, glyphosate and its metabolite, AMPA (aminomethyl phosphonic acid) are amongst the first major pollutants of surface waters [1], [4], and became ubiquitous contaminants in the environment and in tissues of the human body.

The role of adjuvants in pesticide formulations is to aid or modify the action of the active ingredient, and they are typically used in herbicide formulations to exert surfactant effects [5]. Glyphosate is commonly formulated with polyglucosides and polyethoxylated substances as adjuvants facilitating solution of the parent compound in hydrophobic media. A key adjuvants type of the latter group of surfactants is polyethoxylated tallowamine (POEA). The major formulated preparation among glyphosate-based herbicides is Roundup®, in which glyphosate is formulated as isopropylamine salt and POEA is added to enhance herbicidal efficacy by providing better penetrability to plant tissues *via* its detergent effect [1]. Although glyphosate presents lower acute toxicity on vertebrates than other herbicides, it has been evidenced to cause toxicity and genotoxicity in many taxonomic groups, especially in aquatic organisms and amphibians [6]; and to induce endocrine disrupting effects [7], the latter effect being highly synergized by POEA and other surfactants [8], [9] commonly used formulating agents in glyphosate-based herbicide preparations.

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In order to determine the environmental occurrence of glyphosate due to its extensive application systematic monitoring of the active ingredient has been carried out in Hungary (where glyphosate-based herbicide formulations are also market leading pesticides). Moreover, to assess unintended detrimental effects of formulated glyphosate preparations, comparative studies have been carried out with glyphosate, its formulating agent POEA and the formulated herbicide preparation Roundup to measure their effects on an aqueous indicator organism the great water flea (*Daphnia magna* Straus) and on a mouse neuroectodermal stem cell line NE-4C.

II. Materials and Methods

The glyphosate analytical standard used was Pestanal grade, from Riedel-de Haën (Seelze, Germany). Roundup Classic was purchased from public commercial source. Surfactants including POEA were received from Lamberti SpA (Albizzate, Italy). All other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), unless stated otherwise.

A. Immunochemical Analysis of Glyphosate

The level of glyphosate in environmental samples was determined by a commercial enzyme-linked immunosorbent assay (ELISA) method (PN 500086, Abraxis LLC, Warminster, PA, USA) [10], [11] upon chemical derivatization and carried out in 96-well microtiter plates according to a manufacturer-provided protocol. The colorimetric signal obtained in the ELISA system was determined in a Multiskan Ascent microplate spectrophotometer (Labsystems, Finland). Reader developed followed a sigmoid pattern, decreasing with increasing glyphosate concentrations in the samples

B. Immobilization test on Daphnia magna

Aquatic biotests using the giant water flea (*Daphnia magna*) were carried out according to the ISO 6341:1996 standard [12] under controlled photoperiod (16/8 hr light/dark) and temperature (20-22^{o}C), in five repetitions at each datum point, using negative (breeding buffer) and positive ($K_2Cr_2O_7$) controls. Tests were carried out at the first larval stage (6-24 hours) for 48 hours, when the immobilization of the subject animals was recorded (10 animals per test). The sensitivity of the test animals was considered proper according to the standard protocol if the EC_{50} value obtained for potassium dichromate fell in the range of 0.6-1.7 mg/L. Mortality (immobilization) rates were calculated by the Henderson-Tilton formula [13], and EC_{50} values were calculated using probit transformation and log-linear regression.

C. MTT Cell Viability Test

As glyphosate and its formulated herbicide product Roundup has been found to induce apoptosis and necrosis in several human cell types [7] and to exert differential effects in neurotoxicity studies, and were consequently considered to have impacts on neurological development [14], their effects were tested on neural cells. Cells of the mouse embryonic neuroectodermal cell line, NE-4C [15] deposited in the American Type Culture Collection (No. CRL-2925) were used. The cell line originated from primary brain cell cultures prepared from the for- and mid-brain vesicles of transgenic mouse embryos (E 9) lacking functional p53 tumor suppressor protein. NE-4C cells differentiate into neurons and astrocytes in the presence of all-trans retinoic acid, and therefore, provide a suitable model of differentiating neural tissue progenitor cells. Effects on cell viability was measured by NAD(P)H-dependent cellular oxidoreductase enzyme activity in the cells using the MTT reduction test [16].

D. Holographic Microscopy

Cell cultures of NE-4C cells were studied in label-free image cytometry and time-lapse microscopy experiments using holographic transmission microscopy [17], [18]. Cells were cultured overnight in CELLview™ glass bottom cell culture dishes (Cat.-No.: 627870, Greiner Bio One GmbH, Frickenhausen, Germany). Cells were seeded at a density of 5.26×10^4 cells/cm^2 and maintained in growth medium consisting of minimal essential medium (MEM, Sigma Aldrich, Hungary) plus 10% fetal bovine serum (Gibco, Invitrogen Inc., Paisley, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5µg/ml amphotericin in a humidified atmosphere containing 5% CO_2 at 37°C. The stock solutions (1%) of Roundup, POEA and glyphosate were prepared in the cell culturing medium and filtered through a 0.22µm filter. For phase holographic imaging, cells were washed with PBS and culturing medium was changed to assay medium containing the target substances at the required concentrations.

Images were captured at different time points from the different spots on the dish. Three-dimensional structures of cells were visualized in a HoloMonitor M4 digital holographic microscope (Phase Holographic Imaging AB, Lund, Sweden) by sample illumination with 0.1 mW/cm^2 HeNe laser (635 nm). The interference pattern was recorded as a hologram on a digital sensor. Average cell area and thickness were calculated using software HoloStudio M4.

III. Results and Discussion

A. Determination of Glyphosate

The commercial ELISA kit by Abraxis LLC is a convenient method for the analysis of glyphosate both for its analytical parameters (limit of detection (LOD), sensitivity) and for its easy performance. A major advantage of the process is that it is directly applicable on aqueous samples (without any sample extraction step). A fundamental drawback in the analytical sense is, however, in contrast to chromatographic methods, that the ELISA procedure results in a single analytical signal from the sample, providing no detailed information on sample composition, and a technical difficulty is that the signal background was relatively high. The guaranteed LOD of the Abraxis ELISA system is 0.05 ng/ml, the upper concentration limit of the detection is 4 ng/ml (above which sample dilution is required). The method is validated for surface water [10],

[11], [19], and it is highly specific to glyphosate: its cross-reactivity is below 0.1% even for very closely related compounds (glyphosine, glufosinate, AMPA, glycine, etc.).

Using the above method, glyphosate has been detected in 21 surface and ground water samples from 42 samples obtained in a systematic monitoring campaign of pesticide residues in environmental matrices in Békés county (South-East region of Hungary), corresponding to an incidence rate as high as 50%. Detected glyphosate concentrations (Table I) ranged between 0.54 and 0.98 ng/ml with outstandingly high values in 5 and high values in 16 cases.

TABLE I
GLYPHOSATE CONCENTRATIONS IN SURFACE AND GROUND WATER IN BÉKÉS COUNTY, HUNGARY

Code	Sampling site / Type of cultivation	Concentration of glyphosate (ng/ml)
BA2G	Battonya [a]	0.68 ± 0.09
BA3F	Battonya [b]	0.66 ± 0.15
BA3G	Battonya [b]	0.63 ± 0.07
CSF1	Csorvás [c]	0.65 ± 0.13
CSF2	Csorvás [c]	**0.82 ± 0.04**
CS1F	Csorvás [c]	0.68 ± 0.12
KT2F	Kőröstarcsa [a]	0.76 ± 0.04
MH2F	Medgyesegyháza [a]	0.75 0.08
BSZ1A	Békéscsaba [d]	**0.93 ± 0.08**
BSZ1B	Békéscsaba [d]	0.60 ± 0.05
BSZ1E	Békéscsaba [d]	0.66 ± 0.10
GYN1C	Gyomaendrőd [d]	**0.98 ± 0.003**
GYN1D	Gyomaendrőd [d]	0.56 ± 0.26
GYN1G	Gyomaendrőd [d]	0.63 ± 0.04
GYN1H	Gyomaendrőd [d]	0.59 ± 0.05
GYN1J	Gyomaendrőd [d]	0.59 ± 0.11
GYN1K	Gyomaendrőd [d]	**0.87 ± 0.08**
OK1G	Orosháza [e]	0.66 ± 0.04
OK1I	Orosháza [e]	**0.96 ± 0.10**
OK1B	Orosháza [e]	0.58 ± 0.06
OK1M	Orosháza [e]	0.54 ± 0.003

[a] organic (ecological) farming, [b] grassland, [c] intensive agriculture, [d] industrial area, [e] public road services

B. Toxicity to Aquatic Organisms

The occurrence of water contaminating chemicals and their subsequent aquatic toxicity receives special attention, as these microcontaminants enter a matrix that is the habitat of numerous aquatic organisms and the basis of our drinking water resources. Due to the daily contact with water, these contaminants implement chronic exposure. In addition, herbicides can also disturb fresh water microbial communities directly or indirectly and reduce biodiversity in the aquatic community [20].

Negative effects of glyphosate in microalgae and in other aquatic organisms have been commonly reported [21]-[26]. Current studies focused on the effects of the active ingredient or formulation not only on individuals, but also at a community level [27]. Moreover, the effects of chronic sublethal exposure to pesticides may become synergized by other stress factors (e.g. predator stress, competition, abiotic factors) and affect community structure [6], [28].

Formulating surfactants also known to contribute to the toxicological characteristics of herbicide formulations. Glyphosate formulations containing POEA were demonstrated to be more toxic to amphibians [29]-[32] than glyphosate itself, and the given surfactant (assumedly inert) was even found to be the most toxic component [33]-[35]. By contrast in acute test the formulation (Roundup) showed slightly lower toxicity than the active ingredient (glyphosate), however in chronic test this ratio is indicated to be reversed [36]. POEA is assumed to change cell permeability, amplify the effect of biologically active substances through apoptosis and necrosis [7] and to disrupt cell membranes on respiratory surfaces in aquatic organisms [37].

Measurement of acute toxicity on *Daphnia magna* is best defined for glyphosate, determined for the isopropylamine salt of the active ingredient on two *D. magna* populations. Our standard laboratory population (originated from LAB Research Kft., Veszprém, Hungary) showed acute 48-hour LD_{50} values in the 560-1700 µg/ml range (Table II), showing good agreement with literature data. A sensitive *D. magna* subpopulation, selected during breeding from wild type individuals (collected in Pest County, Hungary), however, showed approximately twice as high sensitivity. Roundup was found to be at least 35 times more toxic to the standard laboratory population of *D. magna* than glyphosate its 24-hour LC_{50} value exceeding 20 µg/ml (Table II), also in agreement with manufacturer product documentation data. Assessment occurs to be more complicated in the case of POEA, where literature data indicate toxicity in the 0.1-0.9 µg/ml concentration range depending on the polyethoxylation rate of the surfactant [35]. In our hands, POEA showed no significant mortality at 3.1 µg/ml (the concentration of POEA present in 20 µg/ml Roundup), corresponding to at least 3.7- or 32-fold lower sensitivity than reported [35], when compared to the highest and lowest reference value, respectively.

TABLE II
ACUTE 48-HOUR LC_{50} VALUES OF GLYPHOSATE, POEA AND ROUNDUP ON *DAPHNIA MAGNA* STRAUS

Compound	LC_{50} (µg/ml) determined	LC_{50} (µg/ml) reference
glyphosate	690 (560 – 1700) [a] 900 (750 – 1080) [a] 360 (100-480) [b]	1.4-7.2 [36] 780 – 930 [39], 962 [40]
POEA	> 3.1 [a]	0.097 – 0.849 [35]
Roundup	> 20 [a]	3 (2.6 – 3.4) [21] 3.7-10.6 [36] (R. Classic) 11 [41] (R. Original) 24-37 [42]

[a] standard laboratory *D. magna* colony received from LAB Research Kft., Hungary, [b] laboratory selected wild type *D. magna* population

Cuhra et al. recently reported a detailed study on acute and sublethal chronic toxicity of glyphosate and Roundup on *D. magna* [36]. Their results indicated significantly and vastly higher sensitivity to glyphosate (isopropylamine salt – 1.4-7.2 µg/ml) and Roundup (3.7-10.6 µg/ml), respectively, compared to literature data. In accordance with our observations they also found differences between the sensitivity of various *Daphnia* populations, although their results showed not as

high differences as ours. It remains an unclear question of biology how to interpret the tolerance of *Daphnia* cultures to environmental factors. An extremely high and sometimes contradicting variation of LC_{50} values is seen in literature data. It remains clear, however, that differing sensitivity of *D. magna* subpopulations have to be taken into account when aquatic toxicity of chemicals is assessed. In addition, POEA is known to also exert toxicity due to its common technological purity 1,4-dioxane classified as a 2B possible human carcinogen in the IARC database [38] – that is why only purified POEA containing dioxane at less than 5 ppm level is allowed to be used in formulation industry.

C. Cellular Toxicity

Glyphosate and POEA, acting together, are cytotoxic to human placental, embryonic kidney and liver cell lines at very low sub-agricultural dilutions [7], [43], [44]. This toxicity has mostly been related to endocrine and membrane disrupting effects [7], but neural defects and craniofacial malformations from regions where glyphosate-based herbicides are used have also been identified on amphibian species, and the mode of action has been related to disturbance of the retinoic acid pathway [45]. To assess their separate and combined toxicity, these compounds were applied to model progenitor cells of neural tissues [15]. The viability of cells was determined 2, 6 and 24 hours upon substance administration, in the presence and absence of serum containing 5% bovine serum albumin.

The photometric method to assess cell viability detects the $NADH^+$ and $NADPH^+$ content of the cells through tetrazolium reduction to formazan, and is most suitable to measure metabolic activity, particularly mitochondrial function. Roundup has substantially decreased NE-4C cell viability, while the presence of serum provided a certain "protection" against this cytotoxicity. The effect was detectable already after 2 hours upon administration (Fig. 1 (a)), significantly increased by 6 hours, but no significant further increase was seen until 24 hours. POEA also exerted strong reduction of cell viability: above 0.0005% concentration inhibited cellular metabolism upon even short exposure (2 and 6 hours). Viability in cell cultures containing serum decreased in 24-hour treatments only above somewhat higher POEA concentrations (>0.0026%), indicating a serum-dependent slight tolerance of the cells. In contrast, glyphosate caused substantially lower inhibition of cell viability, as inhibition was seen only above 0.05% concentration. The concentration 1.34% (corresponding to the glyphosate content in 2% Roundup), however, caused full inhibition upon 6 and 24 hours. LC_{50} values on NE-4C cells after 2 hours of exposure, determined by logistic non-linear curve fit from the sigmoid dose-response curves on Fig 1 (a), were found to be 0.013±0.002%, 0.017±0.009% and 6.46±2.25% for Roundup, POEA and glyphosate, respectively (in equivalents of diluted Roundup solution), corresponding to actual concentrations of 0.022±0.003 and 53.1±18.5 mg/ml for POEA and glyphosate, respectively. It has to be noted that the LC_{50} value determined for Roundup is 150-fold lower than concentration (dilution) used in agricultural applications (2%). LC_{50} values indicate equitoxicity between Roundup and POEA, while toxicity of glyphosate occurs over 2 orders of magnitude lower.

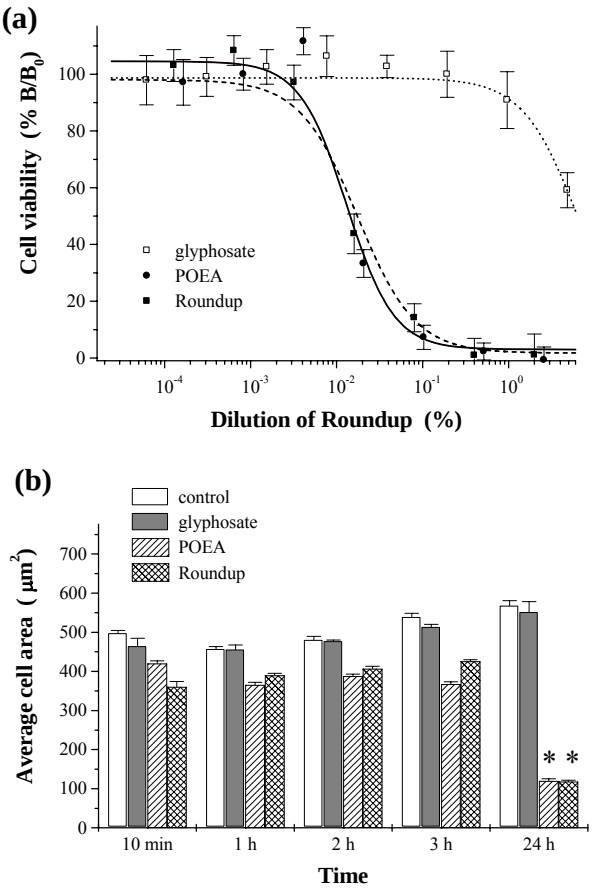

Fig. 1 Effect of exposure of NE-4C cells to glyphosate, POEA and Roundup. (a) Concentration-dependent effects in the MTT test upon 2 hours of toxicant administration. Roundup concentrations correspond to serial dilution of the formulated herbicide. Concentrations of glyphosate and POEA correspond to levels in diluted Roundup solutions. Points on each sigmoid curve are expressed as % of their own background (B_0) value. (b) Time-dependence of cell areas detected in holographic microscopy upon toxicant administration. Roundup, glyphosate and POEA were applied at concentrations of 0.01%, 0.0042% and 0.0016%, respectively (concentrations corresponding to 0.01% Roundup solution). Roundup and POEA caused extensive cell death upon 24 hours of exposure (indicated by * mark)

Cell integrity was visualized by holographic transmission microscopy, a relatively novel, label-free, non-invasive, non-destructive and non-phototoxic method allowing both qualitative and quantitative measurements of living cells over time. In holographic microscopy the illuminating light is split into an object beam and a reference beam. The object beam upon illumination of the object is re-joined and interfered with the reference beam creating a hologram. Focusing within the hologram is possible to any point without any mechanical movement by iteratively created images any time after the actual recording. Cell morphology parameters, determined by holographic microscopy, are useful descriptors of cell viability

and ongoing cell-morphological changes including the processes of cell differentiation, cell growth and cell death.

To further assess how treatments with glyphosate, POEA and Roundup affect cell motility, the sensitivity of NE-4C cells to these substances was characterized by real-time holographic imaging. Cells under toxic effects take rounded shape due to cytoskeletal response, and become detached from surfaces they had adhered to. Consequently a time-dependent decrease in cell area and an increase in maximum thickness of the NE-4C cells were seen in response to treatment. The effect of glyphosate (0.0042%), POEA (0.0016%) and Roundup (0.01%) on average cell area is depicted on Fig. 1 (b). The toxic effect of POEA and Roundup was seen as rapidly as in 10 minutes, followed for up to 24 hours, while glyphosate did not cause statistically significant difference in average cell area compared to the control. Average cell area showed an increase in 24 hours in the control due to cell adhesion, while it was rapidly decreasing due to extensive cell death upon the effect of POEA or Roundup, practically equitoxic with each other at concentrations 20-fold below agricultural application.

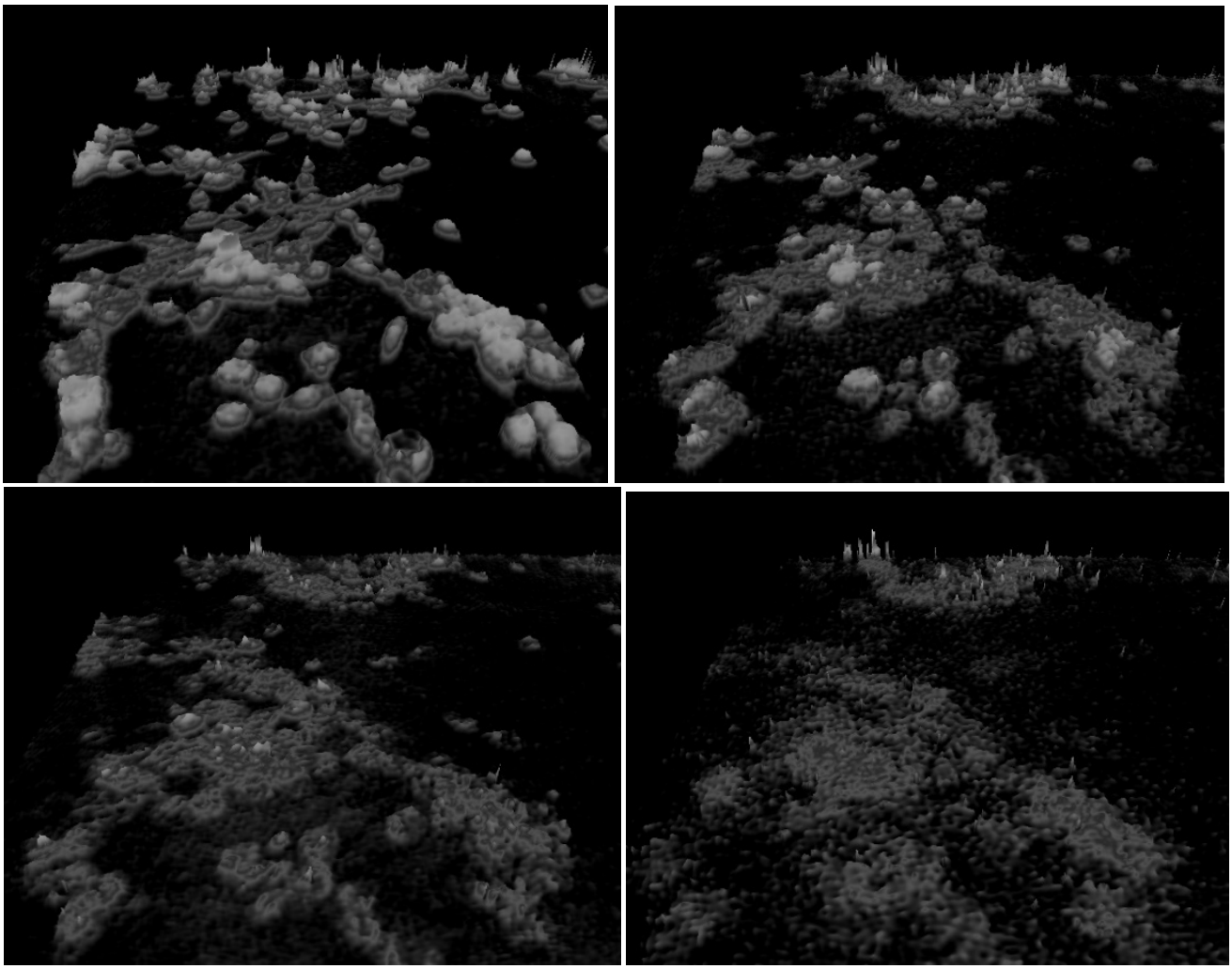

Fig. 2 Time-dependent morphological changes of NE-4C cells exposed to Roundup (0.1%), detected by phase contrast holographic microscopy. Images were captured every five minutes from the beginning of treatment with Roundup (0.1%). After a few minutes of treatment the cells become round, then turn uneven and later break apart

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