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TOXICITY EVALUATION BY FLOW CYTOMETRIC ANALYSIS OF NANOPARTICLES USING THE UNICELLULAR ALGA *CHLORELLA*

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ABSTRACT. The use of nanoparticles for biological and medical applications has rapidly increased and the potential for human and ecological toxicity is a growing area of investigation. For assessing cytotoxicity of nanoparticles we have attempted to assess by flow cytometry the toxicity and ecotoxicity of nanoparticles using the unicellular alga *Chlorella fusca* for developing an in vitro system that could provide an accurate, predictive early screen for assessing the toxicity of different pollutants. Consequently, to understand the mechanisms underlying the toxicity process of nanoparticles we analyzed by flow cytometry the morphological changes (light scattering properties), accompanied by optical and scanning microscopic observations. The investigation showed that analysis by flow cytometry in the system FSC / SSC of *Chlorella* cells incubated for variable in the presence of different dilutions of nanoparticles show induced morphological changes also observed by complementary microscopic techniques. Metabolic activity based on the level of esterases activity with Calcein-AM is a possibility to determine the toxicity of pollutants, including the study of toxicity of nanoparticles prior to their application in various fields of economic life, due to sensitivity and possibility to quantify the results.

Keywords: nanoparticles, flow cytometry, ecotoxicology, metabolic activity, *Chlorella* cells

INTRODUCTION

Nanotechnology is a rapidly expanding and advancing field of research that has already yielded a variety of commercially available products including cosmetics, suntan lotions, paints, self-cleaning windows and stain-resistant clothing. According to conservative estimates (Project on Emerging Nanotechnologies, 2008) the number of consumer products on the market containing nanoparticles (NPs) or nanofibers now exceeds 800 and is growing rapidly.

Despite this increase in the prevalence of engineered nanomaterials, little is known about their potential impact on environmental health and safety (Moore M.N., 2006; Crosera et al., 2009). While risk assessment for human health concerns one species, environmental risk assessment should ideally consider millions of species, with different morphology, physiology and ecological conditions. The current ecotoxicological testing, where a few species are representatives for such diverse groups (crustaceans is of course a gross simplification of an ecosystem (OECD, 1998). Indeed, as no single test or species of living organism show uniform sensitivity to all chemical compounds, a battery of biotests with different sensitivity profiles is recommended and used to assure adequate evaluation of the ecotoxicological situation. Due to the complexity of ecosystems the ecotoxicological hazard assessment is more informative/predictive if the battery involves organisms of different trophic levels (Blaise, 1998; Blinova, 2000; Manusadzianas et al., 2003).

According to recently published review / commentary focusing on the safe handling of nanotechnology has recommended strategic research strategies to support sustainable nanotechnologies by maximizing benefits and minimizing environmental and health risks (Maynard et al., 2006). One of the five key grand challenges cited in the Maynard et al., commentary is to develop and validate alternatives to in vivo toxicity testing of engineered nanomaterials within the next 5–15 years.

In this respect, for assessing cytotoxicity of nanoparticles, Bratosin et al., developed a new experimental cell system based on the use of nucleated erythrocytes (RBCs) from batrachians, which are directly exposed to pollutants or nanoparticles absorbed by different ways and we have evaluated the toxic effects by flow cytometric analysis (Bratosin et al., 2011) .

In this study, we have attempted to assess by flow cytometry the toxicity and ecotoxicity of nanoparticles using the unicellular alga *Chlorella fusca* for developing an in vitro system that could provide an accurate, predictive early screen for assessing the toxicity of different pollutants.

Consequently, to understand the mechanisms underlying the toxicity process of nanoparticles we analyzed by flow cytometry the morphological changes (light scattering properties), accompanied by optical and scanning microscopic observations.

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MATERIALS AND METHODS

Chemicals

In the experiments we chose to compare two types of nanoparticles, one based on porphyrins (P1) and other ferromagnetic composition (P2) both having an important future in numerous medical applications.

Microalgal cultures

Chlorella fusca Krauss et Shihira strain AICB 25 was obtained through the generosity of the National Institute for Biological Science Research & Development (INCDSB), Institute of Biological Research Cluj-Napoca, Algology Laboratory, from their own collection of cyanobacteria and algae. *Chlorella* cells were grown on sterile BBM medium (Bold Basal Medium). All cultures were carried out in sterilized Pyrex glass bottles containing 40 ml of medium. For the assays, the inoculum was taken from a 3-day-old culture, with the aim of using cells growing in a logarithmic phase in all experiments.

In order to evaluate the process of alterations under nanoparticles exposure, cells were exposed to different concentrations of nanoparticles, in serial dilutions, between 0.5 mM and 0.0077 mM, for 24h, 48h and 6 days at 20°C, in culture plates.

Flow cytometric analysis

Flow cytometric analysis were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest Pro software or Cytomics FC 500 for acquisition and analysis. The light-scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale, a minimum of 5000 cells being analysed in each condition. Algal cells size and density were assessed using forward and side-angle scatters (FSC versus SSC).

Morphological changes analysis by scattered light flow cytometry in the mode FSC/SSC

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with the cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter/SSC), on the other hand, correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light

Flow cytometric assay of cell viability using calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin et al., 2005. The membrane-permeable dye Calcein-AM was prepared as a stock solution of 10 mM in dimethylsulfoxide stored at -20°C and as a working solution of 100 µM in PBS buffer pH 7.4. *Chlorella* cells (4 x105 in 200 µl PBS buffer) were incubated with 10 µl Calcein-AM working solution (final concentration in Calcein-AM 5 µM) for 45 min at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of Calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

Optical and scanning electron microscopic analysis.

Direct light microscopic analysis was performed using an inverted microscope equipped with the Olympus BX 43 Olympus VC-30 and the room visualization software CellSens Dimension. ESEM analysis were performed using scanning electron microscope Quanta Fei 250. *Chlorella* cells were fixed for 1 hour with a working solution of 2.7% glutaraldehyde in 0.1M phosphate buffer pH 7.4 and stored at 40C until examination. The pellet was mounted on a Millipore filter nylon 0,45 mm, then the examination room is closed and the examination was performed at a temperature of -3°C, relative humidity of 100% and a pressure of 910 Pa. Working with Spot 4:01 accelerating voltage of 15KV, using GSED detector (gaseous secondary electron detector). Examination time was between 10 to 30 minutes, order the examination in 1500 mag, 3000 mag, 6000 mag.

RESULTS AND DICUSSIONS

Optical and scanning electron microscopic analysis of *Chlorella fusca* under the action of nanoparticles

All samples of *Chlorella fusca* incubated with different concentrations of nanoparticles from sample P2 have been analyzed by optical microscopy, representative results are presented in a comparative manner in Fig 1 for a 24 h incubation. The same samples were analyzed for additional details by scanning ESEM micrograph.

In image analysis it can be seen:

- For the control sample of *Chlorella fusca* representing the start of the culture of microalgae in the experiments (T0), the cells are spherical, very slightly elongated, with a relatively smooth surface and they form aggregated as colonies of cells embedded in an extracellular matrix, colonies of different sizes.
- In all cases incubation witnesses, T24h, T48h and T6 days, cell colonies remain in the extracellular matrix.
- Under the action of ferromagnetic nanoparticles (P2) at high concentrations (P2.1 = 60 mg / ml) colonies diminished the size, appear chlorotic cells, the cell wall is observed to form "cockscombs" and more, could be seen cell of *Chlorella* with formed "protuberances" on surface, phenotype characteristic of apoptotic cells.

Detection of altered morphology by light scattering flow cytometry

The parameters measured by flow cytometry are optical signals whose intensity can be correlated with cell properties. Estimating by measuring changes in cellular absorption and light scattering, also known as "direct analysis system FSS / SSC" (light scatter measurements) is very simple and sensitive. After

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intercepting the incident light, the cell emits a number of signals. Light scattered at an acute angle or shaft (FSC) can be correlated with cell size, thus allowing distinguishing a cell aggregates or cellular debris and assessing cell viability as dead cells diffuses less light in this direction. A right angle light scatter (SSC) allows the study of refractive cytoplasmic morphology and cytoplasmic-ratio.

Analysis of *Chlorella fusca* populations in system FSC/SSC at T0 and after 24h, 48h and 6days is show in Fig 3.

During incubation for 24h, 48h and 6 days for witness of the incubation there was a slight decrease in cell waist and a slight increase in value for the cell contents. Measurement values of XGeoMean switching from 226 (T0), to 211 (T24h) reducing to 208 to T48h and 199.5 after 6 days of incubation under the conditions of the test. For YGeoMean, it is 177 at the time T0, increases at 198 (T24h), to go to 213 (T48h) and 224la T6 days.

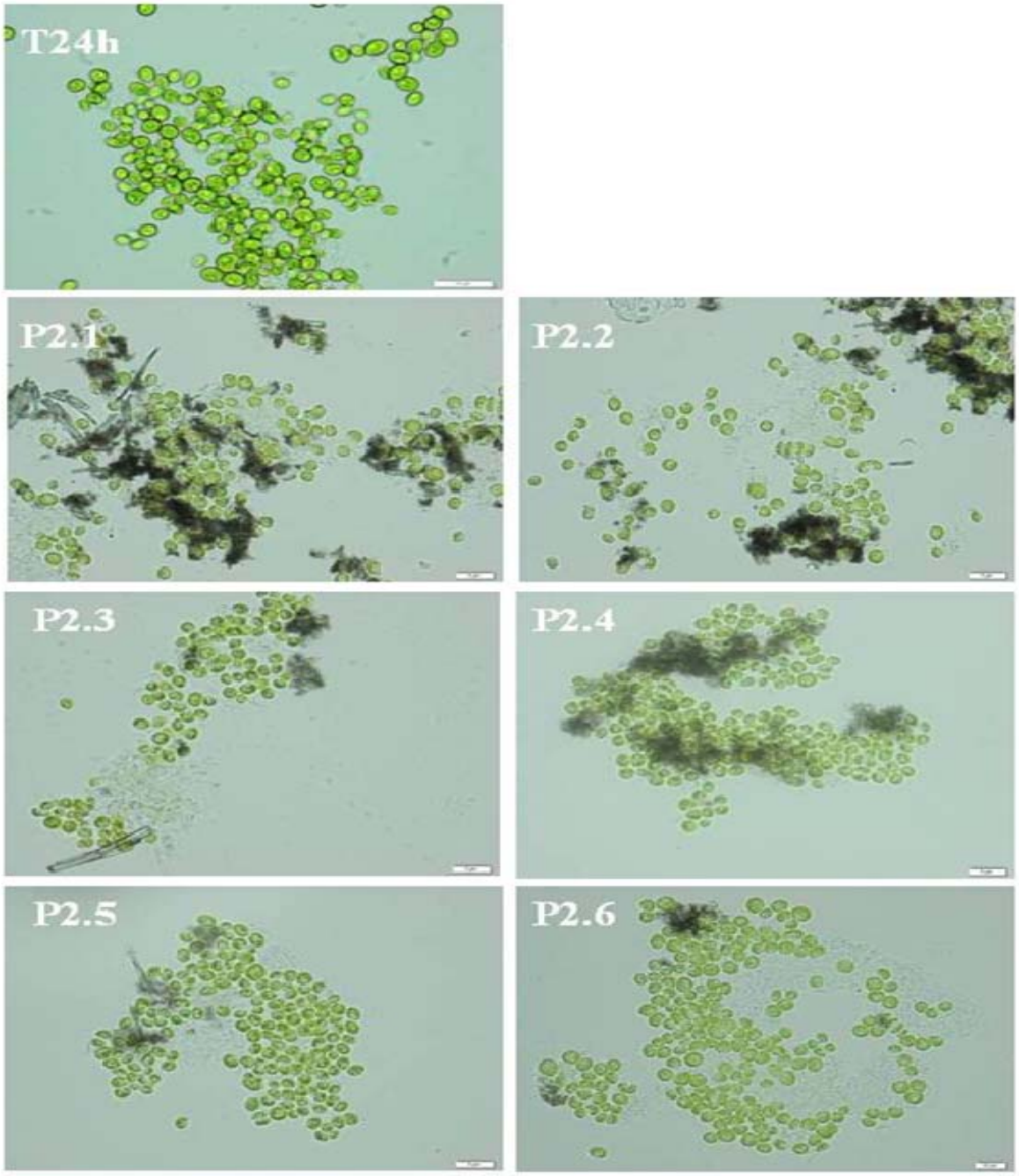

Fig. 1 Analysis by optical microscopy *Chlorella fusca* culture was incubated for 24 hours in the presence of various concentrations (1-6) of nanoparticles (P2) as compared to the control incubation M.

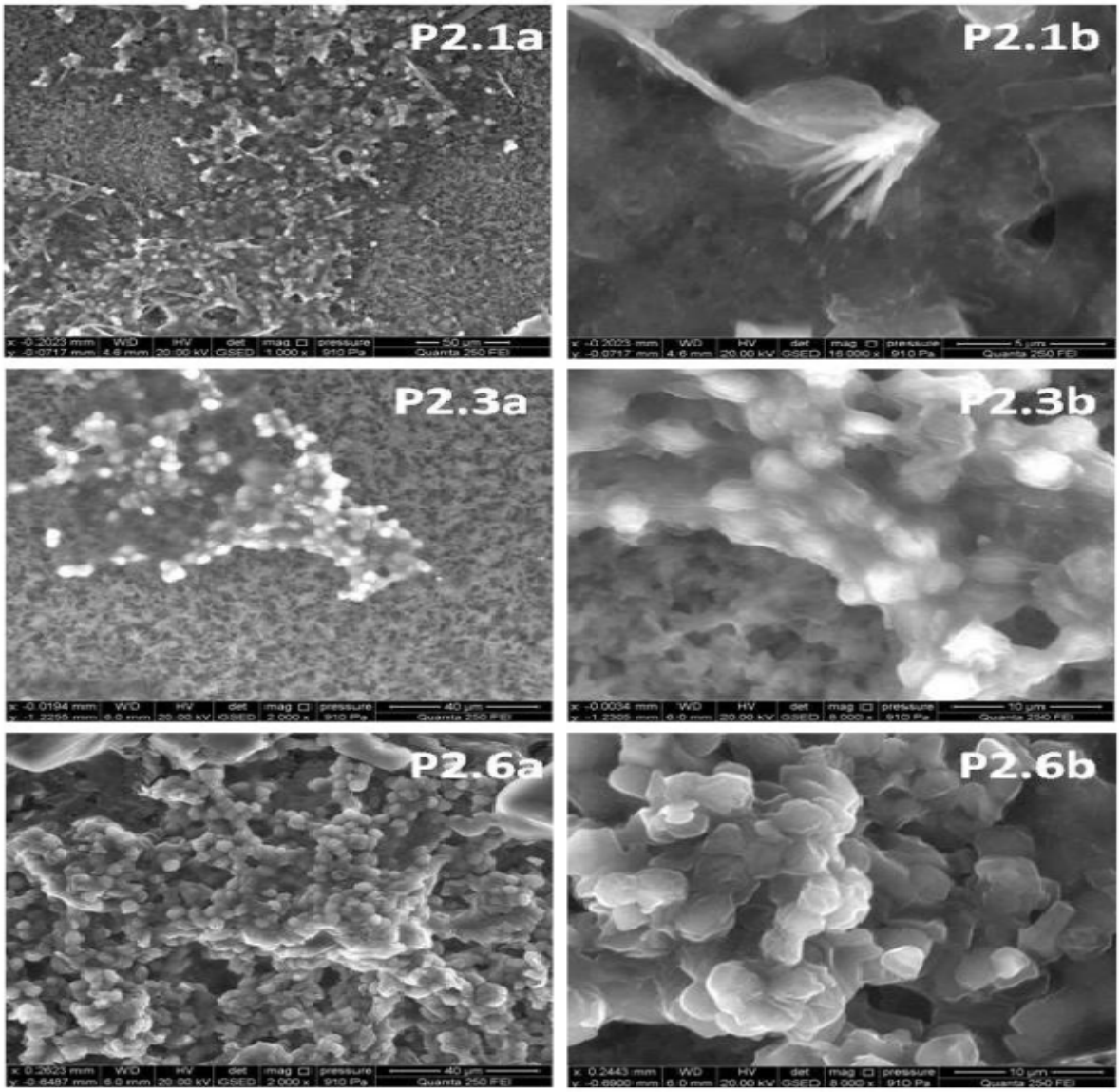

Fig. 2 Analysis by scanning electron microscopy for evidence of morphological changes of *Chlorella fusca* exposed to levels 1, 3 and 6 of ferromagnetic nanoparticles (P2) for 24 hours.

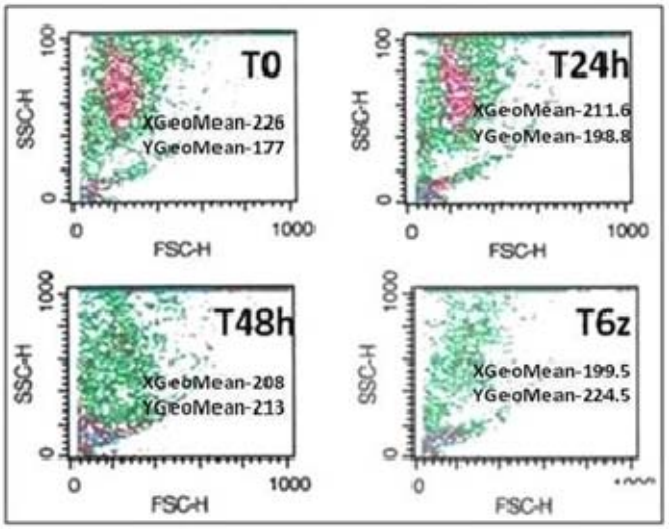

Fig. 3 Comparative analysis in biparametric system (dot-plot) FSC/SSC of *Chlorella fusca* culture at T0 and microalgal cultured for 24h (T24h), 48h (T48h) and 6 days (T6z) days without nanoparticles.

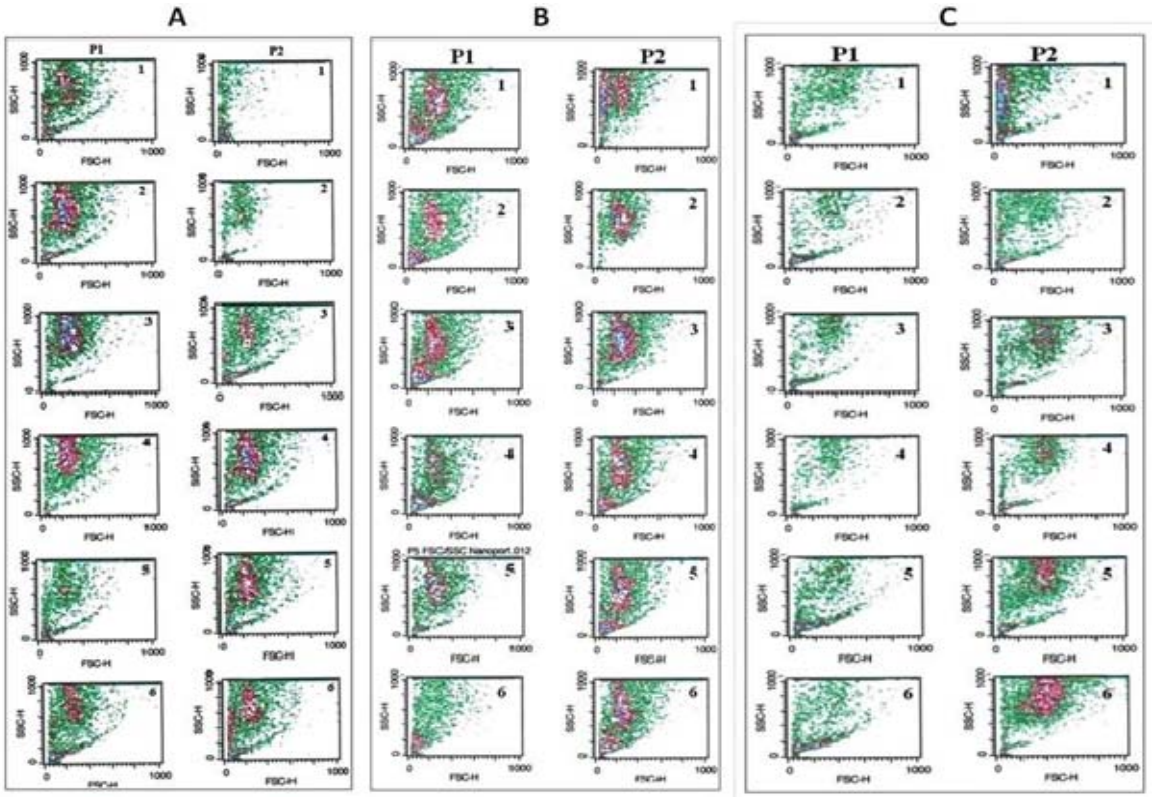

Fig. 4 Comparative analysis in biparametric system (dot-plot) FSC / SSC for culture of *Chlorella fusca* incubated for 24h (A), 48h (B) and 6 days (C) in the presence of various concentrations (1-6) of nanoparticles (P1 and P2).

Influence of porphyrins on cell viability measured with calcein-AM assay

We recently devised a new flow cytometric assay for the measurement of cells viability using Calcein-AM (Bratosin et al., (2005).

The assay is based on the use of acetoxymethyl ester of calcein (Calcein-AM), a fluorescein derivative and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes.

Determination of metabolic activity based on the level of esterases activity with Calcein-AM (cell viability test) translates into average Calcein fluorescence resulting from cleavage of the nonfluorescent substrate Calcein-AM to fluorescent Calcein under the action of intracellular esterases.

As can be seen in fig. 6, a simple incubation for 6 days, lead to a decrease of the fluorescence.

Application of this assay for analyzing the effect of nanoparticles on *Chlorella* cells showed that under the toxic action of the two types of nanoparticles, the different concentrations used in the different times of incubation (24 hours, 48 hours and 6 days) show that MFI values are dependent on the concentration of calcein nanoparticles (Fig. 6), observing a decrease in esterase activity at high concentrations.

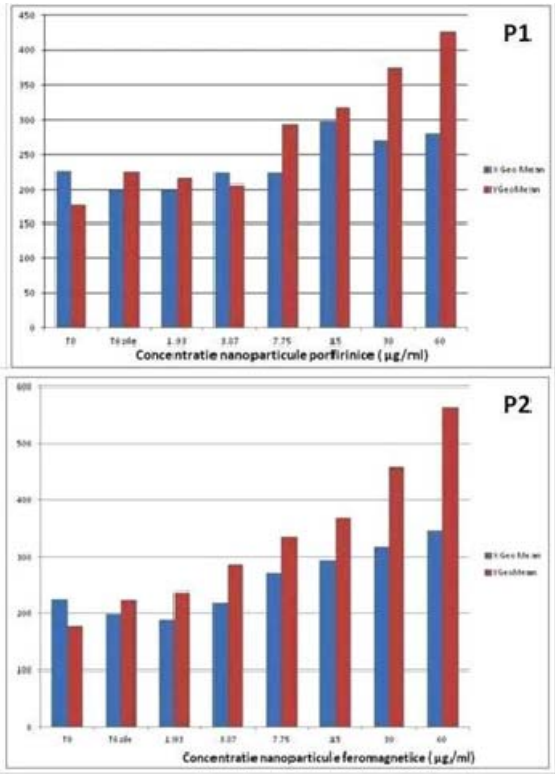

Fig. 5 Comparative histogram for XGeoMean (cell size) and YGeoMean (cell content) of the *Chlorella* samples incubated for 6 days with various concentrations of the dilution series (1-6) of nanoparticles (P1 and P2) as compared to the control (T6 days) and (T0). The values refer to dot-plot analysis of Fig. 4. The results shown are representative of three experiments.

This decrease is much higher under the influence of ferromagnetic nanoparticles from sample P2 compared to the same concentrations of nanoparticles porphyrin which proves that the method is sensitive, allowing a better discrimination of toxicity by applying the test.

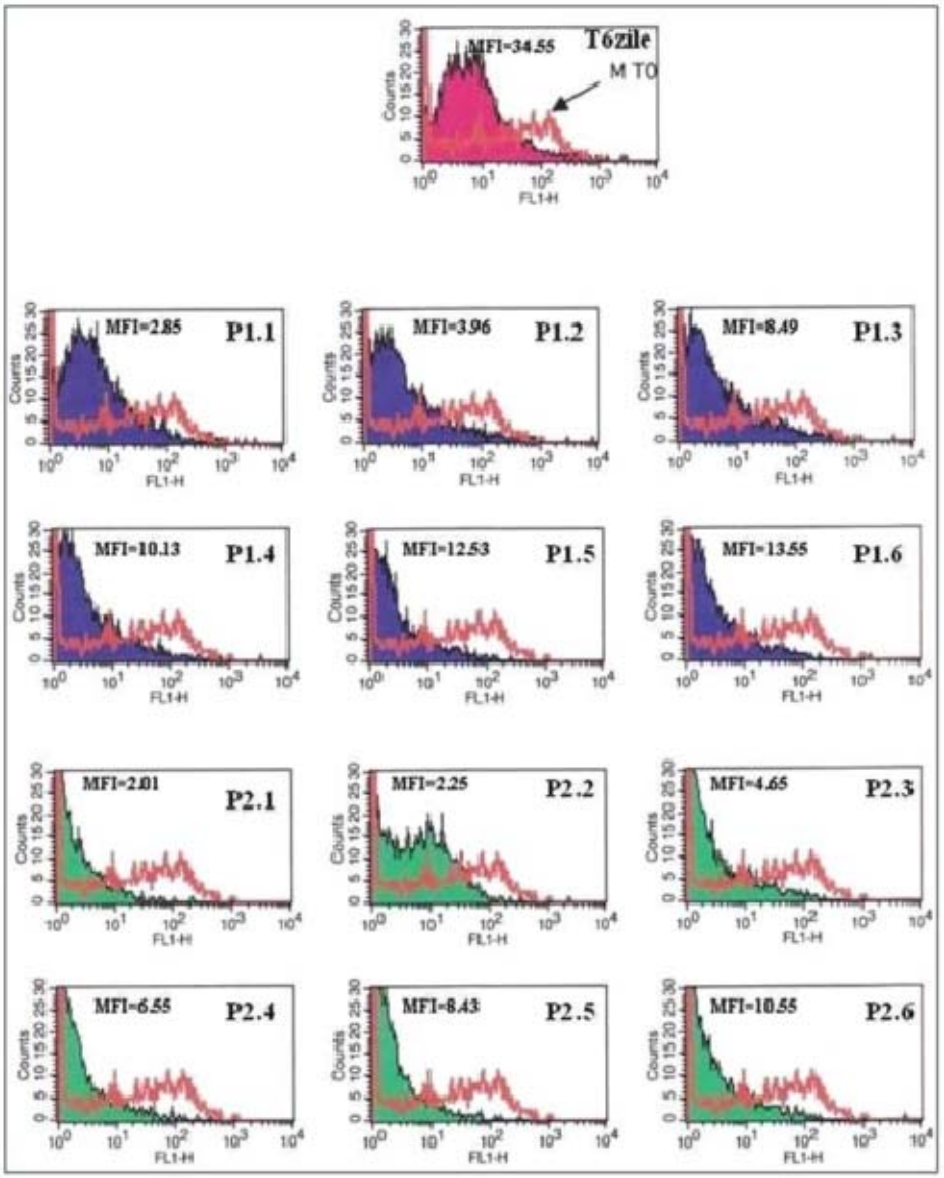

Fig. 6. Comparative analysis of MFI (mean fluorescence intensity) obtained by determining the metabolic activity with Calcein-AM (test cell viability) of *Chlorella* samples incubated with various concentrations of nanoparticles P1 and P2 for 6 days.

CONCLUSIONS

Analysis by flow cytometry in the system FSC / SSC of *Chlorella* cells incubated for variable time (24h, 48h and 6 days) in the presence of different dilutions of nanoparticles show induced morphological changes also observed by complementary microscopic techniques.

Analysis by flow cytometry has the advantage to quantify by value measurements such XGeoMean, YGeoMean, MFI, etc. and a statistical overview of results on a large number of cells (10 000 cells in our case).

Metabolic activity determination based on the level of esterases activity with Calcein-AM is a possibility to determine the toxicity of pollutants due to sensitivity and possibility to quantify the results, including the study of the toxicity of nanoparticles prior to their application in various fields of economic life.

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