

# NUCLEATED ERYTHROCYTES - A NEW EXPERIMENTAL CELL MODEL FOR ASSESSING *IN VITRO* TOXICITY, ECOTOXICITY AND TO DETERMINE THE SAFETY OF FRESH FISH PRODUCTS. A REVIEW

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**ABSTRACT.** The human activities have a negative impact to the environment, consisting in the water contamination with toxic products, heavy metals or with xenobiotic substances. Manufactured nanomaterials (nanoparticles, nanotubes, nanosheets and nanowires) have recent applications in drug delivery, medical devices, cosmetics, chemical catalysts, optoelectronics, electronics and magnetics. Some nanomaterials have been found to be toxic to humans and other organisms either upon contact or after persistent environmental exposure. In present, the measurements of the pollution degree are made with two methods: physico-chemical methods and ecotoxicological test (bioassay or environmental biosensors).

Our results indicate that flow cytometric analysis of nucleated red blood cells viability using calcein-AM and cell death discrimination could provide a rapid and accurate experimental cellular model for effectively screening and evaluating biological responses for *in vitro* nanotoxicology and can be used in ecotoxicology as bioassays for the ecological monitoring of aquatic environment. In the some time, our results indicate that the use of nucleated erythrocytes could be potentially useful for the development of rapid and low cost safety tests to assess fisheries product quality.

**Key words:** nucleated erythrocytes, toxicity, ecotoxicology, pollutants, nanomaterials, apoptosis, flow cytometry

## INTRODUCTION

Environmental pollution is one of the ever surging problems receiving careful attention in our country as well as in the world. The human activities have a negative impact to the environment, consisting in the water contamination with toxic products, heavy metals or with xenobiotic substances. The process of impurification of the surface and underground waters due to the human activities has high dimensions, like the permanent diversification of these toxic substances, was determined by the evolution of the industrial processes. In recent years, some studies have indicated that living organisms are affected from elements present in the environment and the aquatic environment represents the largest sink for accumulation of xenobiotics. In the last decade, eutrophication caused by global industrialisation and anthropogenic impacts on ecosystem can lead to biological damage. Heavy metal analysis demonstrated the presence of nickel, zinc, aluminium and manganese, as a clear demonstration of water quality deterioration. Copper, zinc and iron are trace essential metals for different physiological functions (various enzymes and other cellular proteins), even through their excess can lead to biological damage by excessive intracellular accumulation. Increasing or decreasing levels of these elements in living tissues cause important effects on metabolism.

The revolution in nanotechnology brings advantages in diverse areas of our lives such as engineering, information technology and medicine, etc. (Gross M., 1999; Kim D. *et al.*, 2005; Akerman M.A. *et al.*, 2002). Improvements in nanoscale materials synthesis and characterization have given scientists great control over the fabrication of materials measuring between 1 and 100 nm, unlocking many unique size-dependent properties and, thus, promising many new and/or improved technologies (Oberdörster G. *et al.*, 2005; ASTM E 2456-06, 2006). Recent years have found the integration of such materials into commercial goods and a current estimate suggests there are over 800 nanoparticle-containing consumer products. The production of nanoparticles will increase from 2300 tons produced today to 58000 tons by 2020 (Maynard, 2006). Manufactured nanomaterials (nanoparticles, nanotubes, nanosheets and nanowires) have recent applications in drug delivery, medical devices, cosmetics, chemical catalysts, optoelectronics, electronics and magnetics. Some nanomaterials have been found to be toxic to humans and other organisms either upon contact or after persistent environmental exposure (Oberdörster G., 2004; Zhu S. *et al.*, 2006; Griffith R.J. *et al.*, 2007; Usenko C.Y. *et al.*, 2007).

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 M. *et al.*, 2009). The field of nanotoxicology has formed in response to this lack of informations and resulted in a flurry of research studies. Nanotoxicology is an emerging discipline (Oberdörster E. *et al.*, 2005), a gap between the nanomaterials safety evaluation and the nanotechnology development that produces new nanomaterials, new applications and new products. Nanotoxicology relies on many analytical methods for the characterization of nanomaterials as well as on their impact on *in vitro* and *in vivo* functions (Lewinski N. *et al.*, 2008, Hassellöv M. *et al.*, 2008).

Ecotoxicology has been established in the last two decades as an environmental natural science, evolving on one hand from toxicology, and on the other hand from applied ecology or environmental chemistry.

Ecotoxicology deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects at different levels of biological organisation, from the molecular, cellular, tissue, organ and organism level, up to populations and ecosystems. Ecotoxicological research on selected pollutants requires an interdisciplinary effort, considering physicochemical, molecular, toxicological, physiological and ecological processes. Only an integrated approach considering environmental chemical, toxicological and ecological concepts may be suitable for understanding ecotoxicological effects in contaminated ecosystems

Ecotoxicological research is aimed at an understanding of toxicological phenomena in a variety of biota, populations and ecosystems, and diverse aspects such as mechanisms of toxic action and ecological processes in contaminated systems are considered. Another more prospective approach is based on investigating potential toxicological effects in laboratory assays that may be used for extrapolation to the field.

In present, the measurements of the pollution degree are made with two methods: physico-chemical methods and ecotoxicological test (bioassay or environmental biosensors). The main limits of the analytical methods are the increased expenses of the equipments and the lack of the toxicological informations about bio risk. The biosensor, like a general definition, represents any system which detects the presence of the substratum, by utilization of the biological component which gives a signal, which can be quantified. Cellular biosensors are systems which combine analytical devices and cells to obtain biological signals like recognizing elements. Biosensors have two intriguing characteristics: (1) they have a naturally evolved selectivity to biological or biologically active analytes; and (2) biosensors have the capacity to respond to analytes in physiologically relevant manner. Due their characteristics, these biosensors can detect the variations of the environment and can be used in the ecotoxicology tests and in monitoring of the environment, where pollution source and the nature of the toxic substances cannot be predicted. That's why, the

majority cellular biosensors will be used in monitoring of the environmental toxicity.

Bioassays play a role in this process; however, more comprehensive studies on contaminated systems and ecological and toxicological processes are needed in addition. Often bioassays do not consider the processes in the ecosystem, and neglect environmental factors that influence toxicity. However, they are valuable tools in the characterisation of the toxic action of chemicals, and in the understanding of associated toxicity.

In ecotoxicological research, cellular effect studies are as important as studies in laboratory species because the primary interaction between chemicals and biota occurs at the surface of or in cells. Whether chemical-induced alterations in cell structure and physiology will develop into an adverse toxic effect depends on many parameters, including adaptive responses. The relation between cellular toxicological responses to toxicity at higher biological levels is a key question in ecotoxicology. Hence, cellular toxicology provides an essential concept in understanding ecotoxicological processes, since it plays a key role in elucidating toxic modes of action, and diagnoses toxicological effects at higher biological levels.

In the present is not known exactly the limits of the pollution for human security, of the major ecosystems and of the ecosphere because it is not known the capacity of support of the ecosystems. The pollutions can be much diversified: chemical substances (organically substances, metals, oils, gases); physical factors (heat, noise, radiations, etc) or biological (pathogenic embryo) and they can activate each other, sometimes the establishment of limits concentrations approved is not efficient, even dangerous. In present is trying to find some molecular biomarkers able to measure the risk brought by the water pollution, to the pisciculture food, especially. In ecotoxicology the biomarkers is an obvious change or/and measurable on the molecular, biochemical, cellular, physiological or behavioral level, which shows the actual or the last exposition of an individual on a least one chemical polluting substance. A biomarker measured on the individual level, didn't find the ecotoxicological signification only it is describes, explains and predicts the pollution effects on the populations and communities which develops in their natural environment. They are three kinds of biomarkers: biomarkers of exposition on the xenobiotic, shows that the polluting substances present in the environment penetrate the organism, they been the results of the interaction between pollutants with biological molecules in the tissue and/ or the liquids of the body; biomarkers of the exposure effect allow the demonstration of the fact that a xenobiotic get to the organism and has a toxic effect or notion a critical target (stress biomarkers of sensibility on the effect made by exposure receive the phenomen of variation of genetic origin of the response to the contamination with pollutions and it is translated by a variation of the sensibility (the

increasing of the glutathione S transferase quantity or the decreasing of the sensibility acetylcholinesterase, which can be biomarkers).

The human society, in present, is deal with problems which aim to the quality of life and the safety of the peoples: environmental pollution and the food safety, which are in directly depends.

In this way, the evaluation of the pollution of natural aquatic ecosystems and those from the fish farms especially, and the estimating the risk degree which has on the food, are very important task. In present, the researchers are trying new biological tests for identification of new, sensitive biomarkers for determination of immediate and later effects of different substances on the aquatic environment, in general and on the human health especially. Their identification can lead to the imagination of the cellular biosensors able to monitoring the aquatic ecosystems, according to the actual laws.

To assess aquatic pollution degree or for assessing cytotoxicity or ecotoxicity of nanoparticles, we developed a new experimental cell system based on the use of nucleated RBCs from fishes and batrachians which are directly exposed to pollutants or to nanoparticles absorbed by different ways.

Despite their structural simplicity, the erythrocytes of lower vertebrates preserve nucleus and mitochondria, both the sensors of the PCD machinery. As well as playing a central role in the physiology of respiration, these cells can represented an outstanding model to study xenobiotic-induced damage to different cellular compartments. Little is known about the effect of environmental toxicants on apoptosis induction. The two modes of cell death (apoptosis and necrosis) differ fundamentally in their morphology, biochemistry and biological relevance. We and others have recently shown that programmed cell death (PCD) of nucleated erythrocytes is related to an apoptotic mechanism (Bratosin D. *et al.* 2004).

In our study, to evaluate cell-nanomaterials interactions, nucleated RBCs were exposed to different concentrations of pollutants or nanocomposites and analyzed by flow cytometry, after 24h incubation endpoints for morphological changes (FSC/SSC), apoptosis/necrosis analysis (FITC-annexin-V labeling/PI) and viability (calcein-AM method) or measurement of reactive oxygen species (ROS).

The toxicological analysis were performed comparatively on the porphyrin base or metalloporphyrin and for each porphyrin bare derivative on the correspondent porphyrin-silica-hybrid nanomaterials obtained by sol-gel synthesis in one step acid catalysis or by two steps acid-base catalysis, using tetraethylorthosilicate (TEOS) as silica precursor (Bratosin D. *et al.*, 2011) or , on heavy metal analysis (nickel, zinc, aluminium and manganese).

## DETECTION OF ALTERED MORPHOLOGY BY LIGHT SCATTERING FLOW CYTOMETRY AND MICROSCOPY.

Multiparametric flow cytometric analysis which discriminates and quantifies viable, apoptotic and necrotic cells via measurement of forward and side light scatter (proportional to cell diameter and internal granularity, respectively) is a very rapid and sensible method.

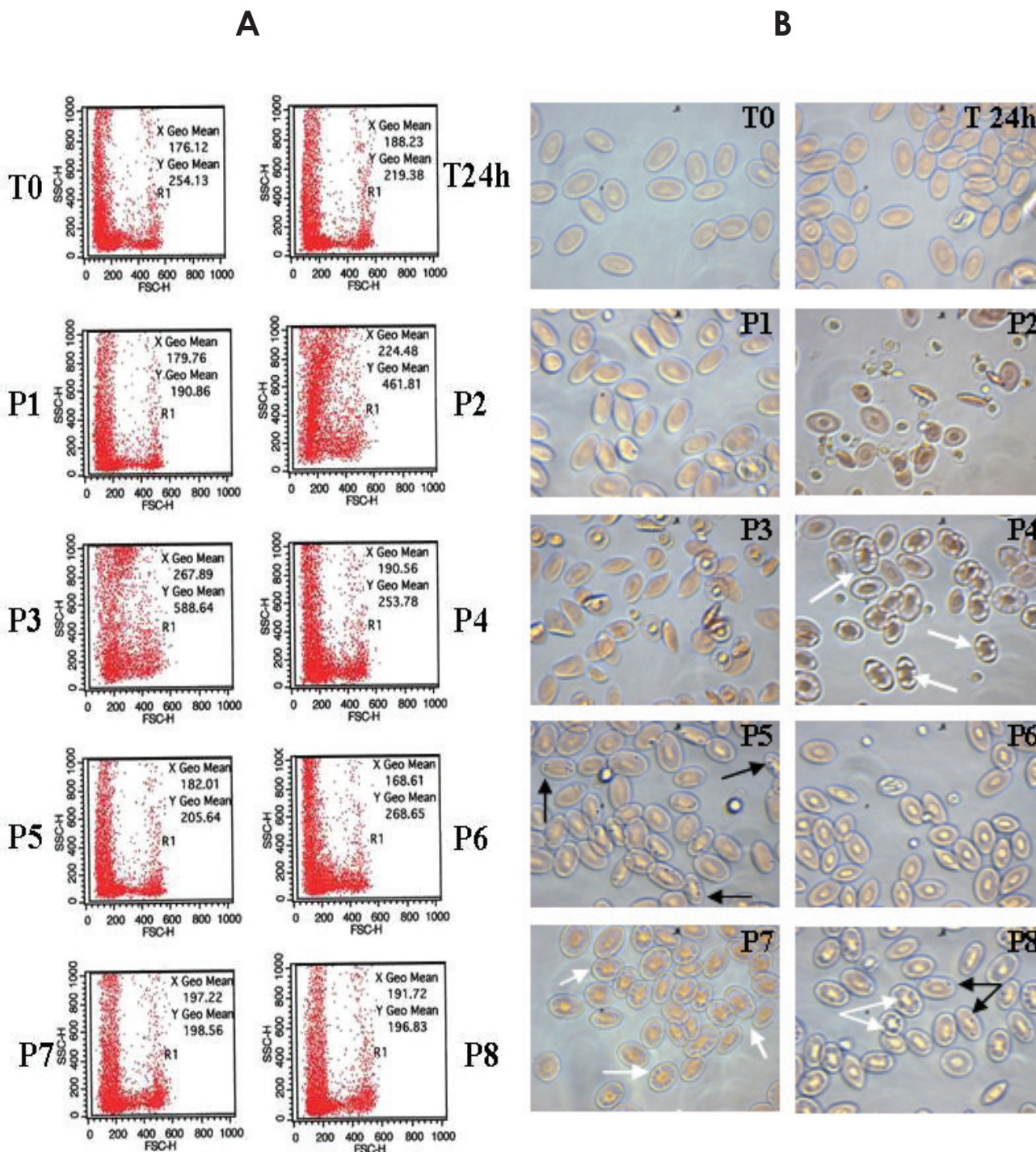
As shown in Figure 1, flow cytometric analysis announce significant morphological changes of nucleated RBCs incubated for 24 h in saline supernatants of different nanomaterials (P1-P8) compared to nucleated RBCs incubated only in saline isotonic solution (T24h).

In fact, the XGeo Mean values (cell side scatter) vary from 168 (P6) to 268 for P3 as compared to the statistical value of normal RBCs, i.e.  $182 \pm 6$ . In the same way, the YGeo Mean values (cell density scatter) vary from 190 for (P1) to 461 (P2) or 588 for P3 as compared to the statistical value of normal RBCs, i.e.  $237 \pm 17$ .

Optical microscopy entirely confirmed these data and showed that morphological changes of nucleated erythrocytes were associated with cell shrinkage (decreased forward scatter and increased side scatter), one of characteristic features of apoptosis. Images of microscopic analyses of nucleated erythrocytes incubated in supernatants obtained by preincubation of nanomaterials in saline solutions show that highlights the morphological changes are not uniform for all samples, neither the intensity nor that the manner of expression, showing that they accurately reflect the toxicity of different samples. Change of discoid morphology to rounded forms, brings to mind an apoptosis phenomenon. They are very numerous in samples P2 and P3, and when they are accompanied by a transparent appearance, providing that these cells are dead. Samples P4 and P7 induce an unexpected morphological aspect, comparable to a "bicycle wheel". Very interesting, in the sample P1 and P5, the nanomaterials produce even more bizarre forms, a sort of "mega pores" or "holes." The same phenomenon is also observed in P8 sample, but less obvious. These morphological changes were confirmed by scanning electron microscopy (Fig 2).

## FLOW CYTOMETRIC MEASUREMENT OF ROS PRODUCTION.

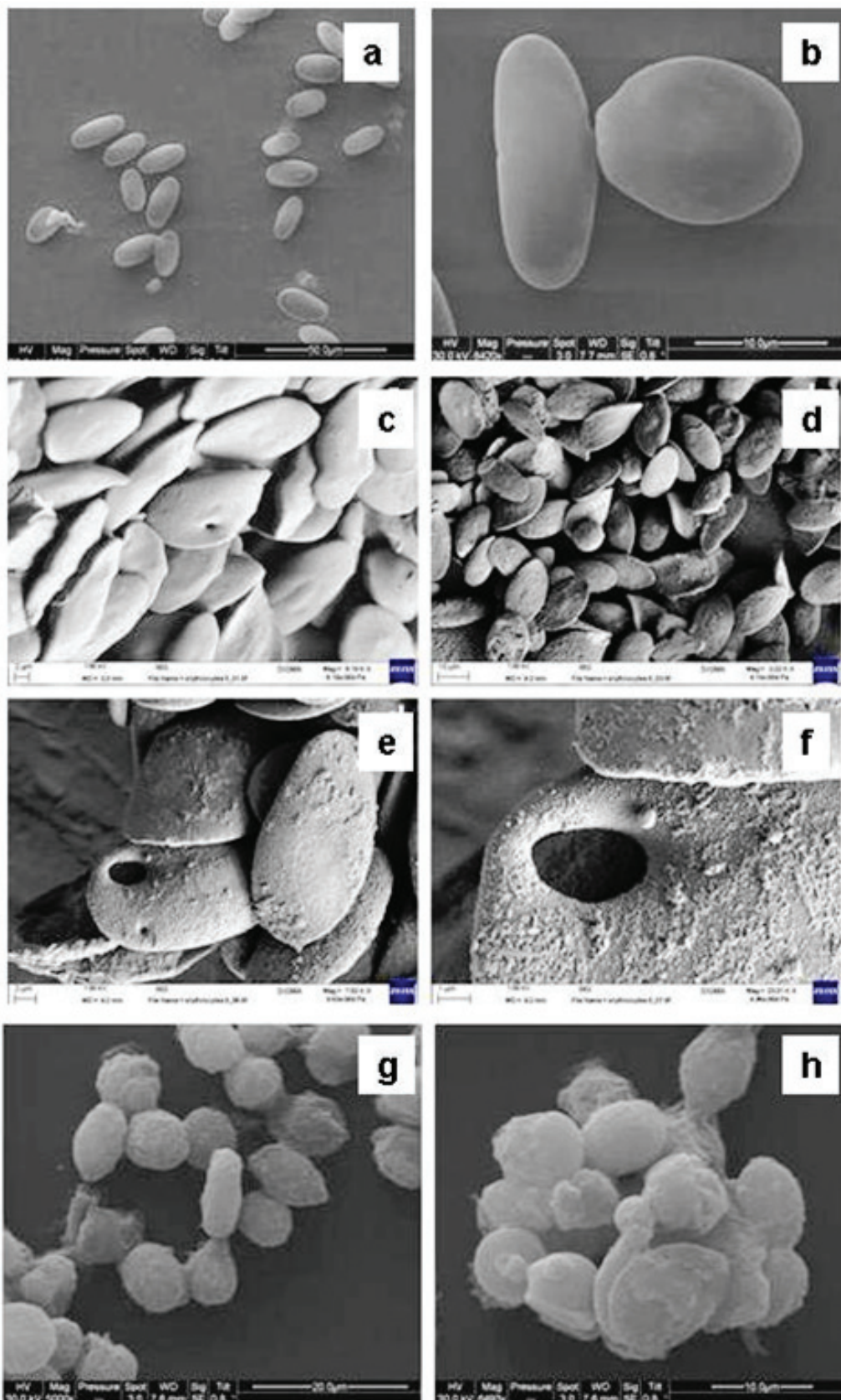
In order to test how aluminium concentrations influences ROS generation, normal nucleated erythrocytes incubated at 20°C for 24h (control normal sample) was compared with erythrocytes stimulated by 2mM  $H_2O_2$  as positive sample (Fig.1A). The calculated average of the MFI values for erythrocytes exposed to various concentration of aluminium showed a significantly higher ability of aluminium to generate ROS as compared to the normal sample (Fig. 3). The MFI for unstimulated normal RBC sample was 34 compared to 48 for normal  $H_2O_2$ -stimulated sample. The results showed that the presence of aluminium caused an increase in fluorescence, between MFI=58 to MFI=79, depending of aluminium concentrations.



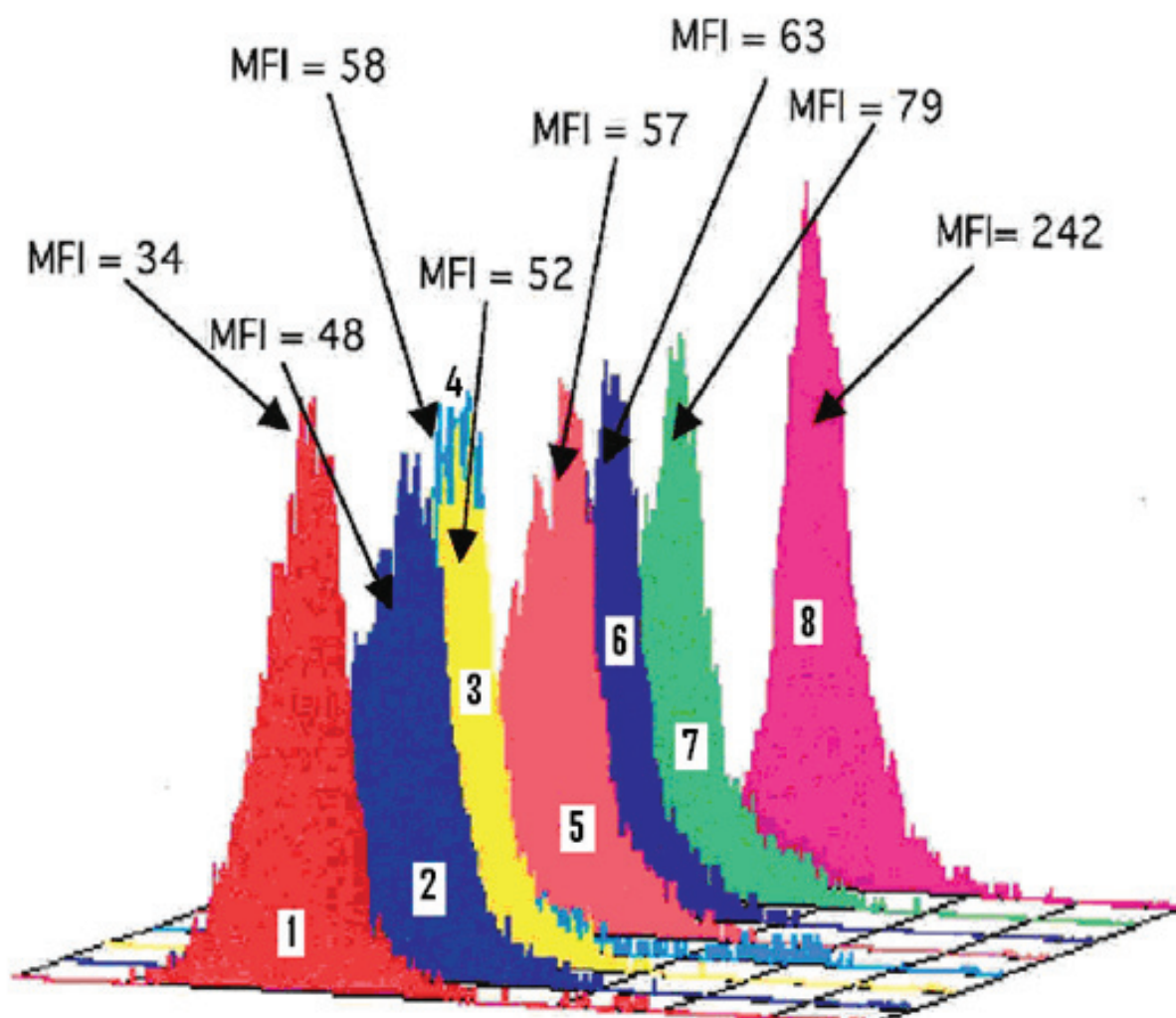
**Fig. 1.** Comparative morphological shape changes analyses by flow cytometry (A) and optical microscopy (B) of normal nucleated erythrocytes (To and T24h) and exposed to nanomaterials (P1 to P8) at 0,008 g/ml. Dot-plot analysis FSC/SSC of cells shape changes. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity and refractiveness).

Data are representative of three analysis giving similar results. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

Black arrows: erythrocytes with “mega pores” or “holes” White arrows: “bicycle wheel” erythrocyte shape. Results presented are from one representative experiment of three performed. Cells were visualized using an inverted microscope MCX 1600 for bright field (Micros Autrich)



**Fig. 2.** Scanning electron microscopic analysis of normal nucleated erythrocytes (a and b) of *Rana* sp., exposed to the action of sample P1 (meso-tetra-tolyl-porphyrin) at 0.008 g/ml (c-f) and to the sample P3 (Zn (II)-meso-tetra-piridil-porphyrin) at 0.008 g/ml (g, h). the results presented are representative experiments.



**Fig. 3.** Comparative histogram of the reactive oxygen species (ROS) produced in red blood cells of *Rana esculenta* under the action of aluminum measured by flow cytometry. 1: red blood cells incubated at 20°C for 24hours, 8: positive control with red blood cells stimulated with 2 mM H<sub>2</sub>O<sub>2</sub>. 1-7: fluorescence of histograms for erythrocytes incubated with increasing concentrations of aluminum. MFI: mean fluorescence intensity of 2', 7'-dichlorofluorescein (DCF).

### INFLUENCE OF PORPHYRINS ON CELL VIABILITY MEASURED WITH CALCEIN-AM ASSAY

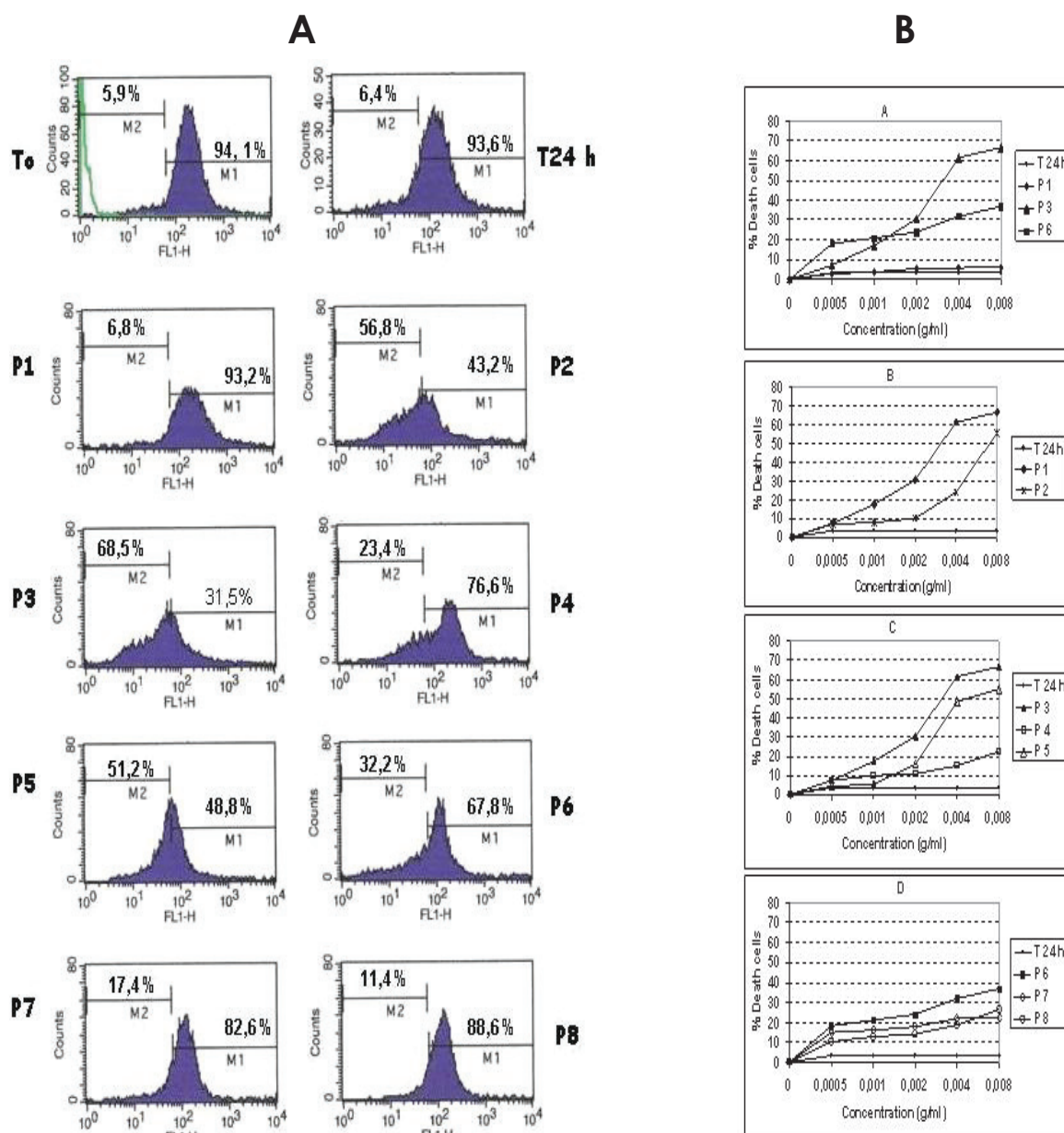
We recently devised a new flow cytometric assay for the measurement of cells viability using calcein-AM (Bratosin D. *et al.*, 2005). The assay is based on the use of acetoxymethyl ester of calcein (calcein-AM), a fluorescein derivative and nonfluorescent vital dye that passively crosses the cell membrane of viable cells and is converted by cytosolic esterases into green fluorescent calcein which is retained by cells with intact membranes. In this regard, it is important to mention that we have previously demonstrated that the loss of esterase activity was an early event that occurred before phosphatidylserine exposure (Bratosin D. *et al.*, 2005).

Application of this assay for analysing the effect of nanomaterials practised on nucleated erythrocytes showed

that two regions could be clearly and unambiguously defined: the region of fluorescent erythrocytes with intact membranes that is related to intracellular esterase activity and strongly correlated with the number of living cells (region M1) and the region of nonfluorescent dead cells with damaged cell membranes (region M2).

As shown in Figure 4, the number of viable cells (region M1) in population decreased drastically as an expression of toxicity of nanomaterials especially for P3 (around 31.5%) or P2 (around 43.2%) as compared to normal erythrocytes population (around 94%).

To get an evident grasp of nanomaterials toxicity, a quantitative dose-response curve was adopted for comparison. For this reason, this test can be a test of toxicity or eco-toxicity, allowing us to determine EC<sub>50</sub> (Figure 4).



**Fig.4.** A:Comparative flow cytometric histogram analysis of calcein-AM cell viability of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml nanomaterials ( P1 to P8). M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Abscissae: log scale green fluorescence intensity of calceine (FL1). Ordinates: relative cell number. B: Curves dose-response for the calculate of EC<sub>50</sub>. Abscissae: concentration of nanomaterials. Ordinates: % of death cells corresponding of M2 region from flow histograms presented in Fig. 4 A. Results presented are from one representative experiment of three performed. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

### STUDY OF ERYTHROCYTES DEATH BY ANNEXIN V-FITC AND PROPIDIUM IODIDE DOUBLE-LABELLING

To investigate the mode of cell death induced by porphyrins, we applied simultaneous staining of erythrocytes with annexin-V and propidium iodide. Normal and incubated erythrocytes were analyzed by flow cytometry for phosphatidylserine (PS) exposure (Annexin-V labelling) and membrane permeabilization (PI-labelling). Phosphatidylserine residues are exposed in the external leaflet of cell membrane early during the process of apoptosis whereas the uptake of propidium iodide indicates a disrupted cellular membrane integrity generally observed during late apoptosis and cell necrosis.

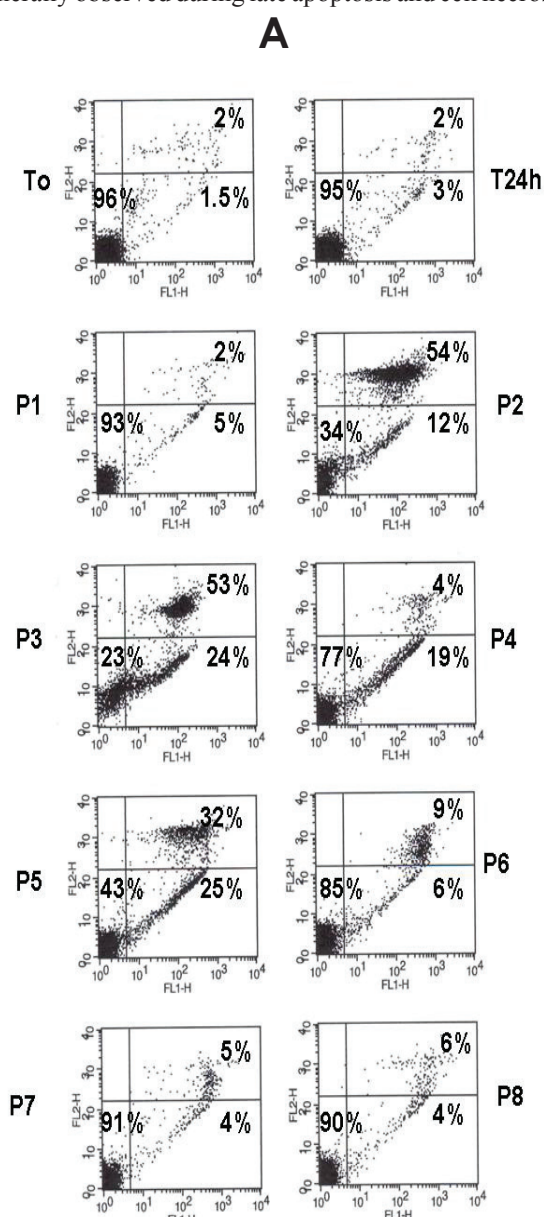
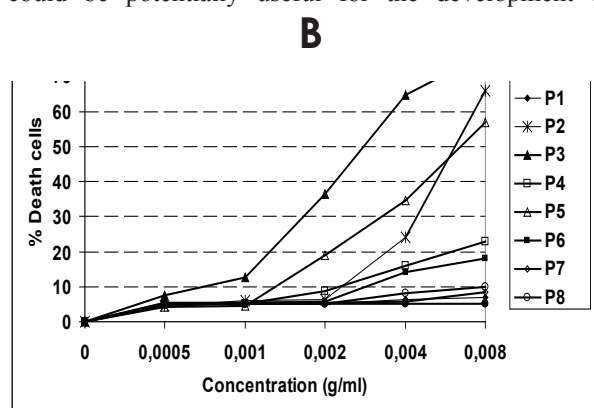


Figure 5 shows comparative flow cytometric analyses of normal (N) and incubated erythrocytes with porphyrins. The number of living cells (Annexin<sup>-</sup>/PI<sup>-</sup>) decreased drastically from 96% (normal erythrocytes) to 23% for P3.

As shown in Figure 5, we can see that porphyrin base or porphyrin-nanomaterials has *in vitro* serious deleterious effect on nucleated erythrocytes in a dose-dependent, allowing calculating EC<sub>50</sub>.

Our results demonstrate that nucleated RBCs can be a new experimental cellular model easy to use, with no costs for culture and for maintaining in the culture. Our results indicate that the sensitivity of nucleated RBCs to nanomaterials was further increased and the information could be potentially useful for the development of



**Fig. 5. A:** Comparative flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml nanomaterials (P1 to P8). Abscissae: log scale green fluorescence intensity of annexine-V-FITC (FL-1). Ordinates: log scale red fluorescence intensity of propidium iodide (FL-2). Low left quadrant: viable cells (annexin-V and propidium iodide negative cells); low right quadrant: apoptotic cells (annexin-V positive and propidium iodide negative cells); upper right quadrant: dead cells (annexin-V and propidium iodide positive cells). % refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed. **B:** Curves dose-response for the calcule of EC<sub>50</sub> conforming to % of death erythrocytes determined by Annexin V-FITC and propidium iodide double-labelling. Abscissae: concentration of nanomaterials. Ordinates: % of death cells refers to the % of total cells (100%) less % of viable cells (low left quadrant: viable cells (annexin-V and propidium iodide negative cells) from flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained presented in Figure 6. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.



low cost and rapid ecotoxicity assays and erythrocyte apoptosis can be an efficient ecotoxicological biomarker providing significant information of environmental stress.

The simplest test applied for evaluating the cytotoxicity of single-walled carbon nanotubes and gold nanoparticles involved inspection of the cells with bright-field microscopy for assessment of cellular and nuclear morphology (Fiorito S. *et al.*, 2006; Altman S.A. *et al.*, 1993; Bottini M. *et al.*, 2006; Goodman C.M. *et al.*, 2004). However, most cytotoxicity assays used measure cell death via colorimetric methods by measuring plasma membrane integrity and mitochondrial activity. (Borenfreund E., 1985; Flahaut E. *et al.*, 2006; Monteiro-Riviere N.A. & Inman A.O., 2006) and the most widely used test is the MTT viability assay (Flahaut E. *et al.*, 2006; Monteiro-Riviere N.A. & Inman A.O., 2006; Jia G. *et al.*, 2005; Tian F.R. *et al.*, 2006; Sayes C.M. *et al.*, 2009).

A third cytotoxicity assay used in several carbon-nanoparticle studies is lactate dehydrogenase (LDH) release monitoring (Sayes C.M. *et al.* 2004; Muller J. *et al.*, 2005). Other cytotoxicity assays determine the genotoxic potential of nanoparticles measuring the extent of DNA damage by flow cytometry using a membrane-impermeable dye (Kastorelos K. *et al.*, 2007; Cui D. *et al.*, 2005) or by comet assay in individual cells using gel electrophoresis (Fairbairn D.W. *et al.*, 1995). The major biological effects involve interactions with cellular components such as the plasma membrane, organelles or macromolecules, but, because the different nanoparticles can trigger distinctive biological responses, it is very important that cytotoxicity studies are conducted for each nanoparticle type (Lewinski N. *et al.*, 2008) with more different cell types.

The nanoparticles and nanomaterials could be absorbed by respiratory tract (inhalation), digestive tract (ingestion) and dermal (penetration) and were subsequently distributed as noted in such key organs as lung (Warheit D.B. *et al.*, 2007), lymph nodes (Bermudez E. *et al.*, 2004), liver (Wang J.X. *et al.*, 2007), brain (Thomas C.L. *et al.*, 2006).

As we know, blood plays a vital role in carrying oxygen from lungs to tissues or organs to meet metabolic needs, erythrocyte, dominant (99%) cell in the blood, can be vulnerable to toxicity (Rothen-Rutishauser B.M. *et al.*, 2006) and the erythrocytes treated with nano-TiO<sub>2</sub> presented morphological change from biconcave shape and underwent abnormal sedimentation, hemagglutination and dose dependent hemolysis (Li S.Q. *et al.*, 2008). Unfortunately, up to now, nothing was known about the interaction of nanoparticles or nanomaterials with nucleated erythrocytes from fishes and batrachians which are directly exposed to pollutants or to nanoparticles absorbed by different ways.

## NANOPARTICLES AS POTENTIAL AQUATIC POLLUTANTS; IMPACT OF OUR RESULTS ON AQUATIC ENVIRONMENT

The nanotechnology industries start to come on line with larger scale production, it is inevitable that nanoscale products and by-products will enter the aquatic environment (Moore M.N. *et al.*, 2004). Consequently, environmental release of nanoparticles into aquatic systems rise many questions, mainly what will be the implications of nanoparticle exposure for organism health and ecosystem integrity? Other question is if the particle size and surface properties will be significant factors in determining toxicity and pathogenesis of nanoparticles in aquatic organisms?

Uptake of nanoparticles in aquatic animals includes direct ingestion or entry across epithelial boundaries such as gills, olfactory organs or body wall. Recent studies with fish have indicated that C60-fullerene may be internalized by these routes, although this was a very limited investigation (Oberdörster E., 2004).

Predicting the behaviors of nanoparticles is likely to be much more difficult than predicting those of conventional chemical pollutants, which is still often a major challenge. Consequently, until we can effectively discount specific or generalized hazards associated with various types of nanoparticle we should invoke a precautionary approach (Colvin V.L., 2003; Howard C.V., 2004). This will require testing of existing and new nanomaterials to determine individual level impacts on animal health status.

In our paper, from this series of experiments, we can conclude that studied porphyrin base or porphyrin-nanomaterials has seriously deleterious effect on nucleated erythrocytes in a dose-dependent *in vitro*, and consequently the erythrocyte is extremely vulnerable.

To emphasize the toxic effect of nanoparticles we examined cell viability and consecutively apoptotic processes. Particular attention has been accorded to evaluation of porphyrine or porphyrine-nanomaterials action in relation to initial and late apoptotic phases and with cell viability measurement using calcein-AM by flow cytometry.

The results reported in the present study indicate that the exposure of nucleated erythrocytes to nanomaterials induces a dependent apoptosis cell death. The changes of all the erythrocyte parameters investigated appear to be strongly correlated with increasing concentration of nanoparticles or nanomaterials and flow cytometric analysis of nucleated RBCs viability and cell death discrimination could provide a rapid and accurate analytical tool for evaluating *in vitro* the biological responses towards of nanoparticles, for assessment of toxicity and biosafety of nanomaterials and environmental nanotoxicity.

The higher level consequences for damage to animal health, ecological risk and possible food chain risks for humans, lead to the need generally applying toxicity and



ecotoxicity testing protocols to identify harmful effects associated with nanoparticles.

## CONCLUSION

The programmed cellular death was considered an uncontrolled degenerated phenomenon in the body homeostasis like response to a cellular aggression. In the last years was discovered that the all cells of the body have the ability to activate a programme of cellular death called apoptosis, which is changing the original idea and lead to a revaluations of all biological aspects including immunology, development and most recently toxicology. The specialists in toxicology considered for a long time of period, that the cells can be killed by great variations of chemical substances or pathological conditions, which in higher concentrations gives cellular lesions or a disturbance of the cellular medium and cellular explosions.

Apoptosis is very important for the molecular toxicology, having a central role in the action of many toxic substances. In these conditions, apoptotic biomarkers can provide informations about the aquatic pollution degree. A special attention is placed on the determination of the oxidative stress of the action of pollutants by measure the reactive species of oxygen (ROS) by flow cytometry, with the advantage that although has a short time and hard to dose, by this method they are intracellular identification with a high (Bratosin D. *et al.*, 2007).

Now, a major challenge for ecotoxicologists will be the derivation of toxicity thresholds for nanomaterials and determining whether or not currently available biomarkers of harmful effect will also be effective for environmental nanotoxicity and new methods are required to assess the toxicity and ecotoxicity of nanomaterials.

The results reported in the present study indicate that our new flow cytometric protocols can be used to create dose-response curves which allow us to determine EC50 for toxicity or eco-toxicity tests. Also, this new tests are generally applicable for identifying harmful effects associated with general antropic impact for the aquatic environment and for its biomonitoring, and finally, with consequences for environmental protection. Our findings suggest that the risk assessment of nano-materials and nano-products with nucleated erythrocytes should be carried out prior to their wide application although they hold much more inconceivable and remarkable advantages.

Our results indicate that flow cytometric analysis of nucleated red blood cells viability using calcein-AM and cell death discrimination could provide a rapid and accurate experimental cellular model for effectively screening and evaluating biological responses for *in vitro* nanotoxicology and can be used in ecotoxicology as bioassays.

At a time when major advances in science, technology and health care offer real possibility of improved health status, and opportunity to many, it is

uncomfortable to realize that environmental factors continue to make a major contribution to sickness and morbidity on a global scale.

During the last two decades, substantial efforts have been made towards the development and international acceptance of alternative methods to safety studies using laboratory animals. In the some time, the use of flow cytometric analyses of fish erythrocytes could be an alternative system for the ecological monitoring of aquatic environment Our results indicate that the sensitivity of nucleated erythrocytes to toxicants was increased and the information could be potentially useful for the development of rapid and low cost safety tests to assess fisheries product quality.

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