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FLOW CYTOMETRY OF NUCLEATED RED BLOOD CELLS USED AS MONITORING TECHNIQUE FOR AQUATIC RISK ASSESSMENT. A REVIEW.

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ABSTRACT: During the last decades anthropogenic factors led to a significant enhancement of pollutants in aquatic environment and for several years, chemicals analysis has been commonly employed. These techniques cannot detect and quantify many environmental phenomena such as bioavailability, bioaccumulation and synergistic effects. For these reasons, many investigations for evaluating the effects of xenobiotic on organisms use *in vitro* or *in vivo* bioassays. The bioassays give a global response for all chemicals present in the environment and these represent one of the best ways to estimate the risk assessment of pollutants in environment for monitoring.

For assessing cytotoxicity or ecotoxicity of pollutants (heavy metals, nanoparticles, etc.) and to assess aquatic pollution degree and biomonitoring of Danube River and Danube Delta, we developed a new experimental cell system based on the apoptosis of nucleated erythrocytes from fishes and batrachians which are directly exposed to pollutants absorbed by different ways. Despite their structural simplicity, the erythrocytes of lower vertebrates preserve nucleus and mitochondria, both the sensors of the programmed cell death (PCD) machinery to develop an apoptosis phenomenon. Our proposed bioassays which are based on the apoptosis phenomenon as induced biomarker by pollutants on fish or amphibians erythrocytes, evidenced by flow cytometry (apoptosis/necrosis discriminated by FITC-annexin-V labeling/PI and cellular viability measured with calcein-AM method) could be rapid and very sensitive tests for in laboratory aquatic risk assessment and biomonitoring. Standardization and application of these tests will surely provide the opportunity of their use easily in ecotoxicological laboratories, biomonitoring of large river basins such as the Danube River Basin and will be also able deliver information on fish as a food product.

Keywords: nanoparticles; nucleated erythrocytes; apoptosis; cell viability; flow cytometry; toxicity; ecotoxicology; nanomaterials.

INTRODUCTION:

"During my travels to the four corners of the world, I often felt to arrive just in time. Just in time to discover the relics of a world that is vanishing. Our Earth has reached an unprecedented level of vulnerability, and species are at risk today to "ultimate extinction." Out of this chaos that we may cause, we cannot imagine for one second only draw our cards Thurs and we lie to ourselves. Aware that our planet is fragile, it is now tired of suffering the repeated assaults of man, it is already a big step in a new direction. Romanian philosopher Cioran said: "Man is an animal who betrayed, and the history will punish him". Can we all do not give him justice?!"

"It is more than time to react if we do not want tomorrow - decision makers and politicians, but also each of us - to be accused by history of helplessness of the imperiled planet", said Nicolas Hulot, Founder and President of the "Foundation Nicolas Hulot for Nature and Man" (Fondation Nicolas Hulot pour la Nature et l'Homme) journalist, producer and an entertainer of TV's "Ushuaia Nature" [Hulot (2005)].

The preoccupations related to pollution and environmental protection at the end of twentieth century and the beginning of the third millennium gives a major interest in scientific research field to European and global level, because these are related to another major objective which follows biodiversity protection. More recently it was found that anthropic pollution has certainly an impact to long-term on the health of human population and perhaps even on human evolution as a species.

The human activities have a negative impact to the environment, consisting in the water contamination toxicants, toxic products, heavy metals, nanoparticles or with recalcitrant 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 determined by the evolution of the industrial processes.

Currently it 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

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capacity to 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, being even dangerous.

Over the past decade, eutrophication caused by global industrialisation and anthropogenic impacts on ecosystem can lead to biological damage. 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. Heavy metals 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 [Alessia et al., (2007)]. Increasing or decreasing levels of these elements in living tissues cause important effects on metabolism.

Given the further contamination of the biosphere by increasing amounts of most pollutants of various origins, it appeared the ecotoxicology as an environmental natural science which studies the interactions between environmental chemicals and biota at different levels of biological organization, from the molecular, cellular, tissue, organ and organism level, up to populations and ecosystems. Ecotoxicological research requires interdisciplinary approaches to study physicochemical, molecular, toxicological, physiological and ecological processes, focusing on adverse effects. 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 pollution degree are made with two methods: physico-chemical methods and ecotoxicological assays (environmental biosensors or bioassays). The major limits of the analytical methods are the cost for the use of analytical equipment and their higher expenses with modern equipments, the lack informations about transformation, bioavailability, synergistic or antagonistic products and 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, this being quantified. Cellular biosensors are systems which combine analytical devices and cells to obtain biological signals like recognizing elements. *Biosensors have two very important 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.* On the other hand, in ecotoxicological assessments bioassays (ecotoxicity tests or biotests) are one of the main tools, defined as methods which use living cells, tissues, organism or communities to assess exposure-related effects of chemicals [Fent (1998)]. The scope of the bioassays is very wide, including testing systems at the molecular level to the ecosystem level: 1) tests with sub-cellular complexes, cells, tissues and organs, 2) tests with whole organisms of one species (mono-species tests) and its population, or, 3) multi-species tests as micro- or mesocosm.

Most common are tests on the organisms level, so-called mono-species tests [Forbes & Forbes (1994)]. Furthermore, bioassays can be distinguished according to the duration of exposure (in relation to the duration of the life-cycle of the test species) as acute, sub-chronic (prolonged) and chronic. For most of the vertebrates and invertebrates acute toxicity tests take a maximum of 96 hours. Sub-chronic (prolonged) exposures generally cover less than one reproduction cycle, while chronic exposures continue over one or more life-cycles. With sub-organism systems can be detected the modes of action of substances and if basal cytotoxicity [Kristen (1996)] or key functions of living matter [Grimme (1993)] are affected, they can give valuable information for possible consequences on the ecosystem-level.

The bioassays have the capacity to assess toxic potentials of chemicals or mixtures of chemicals (acute toxicity, genotoxicity, etc.) often bioassays do not consider the processes in the ecosystem and neglect environmental factors that influence toxicity. More comprehensive studies on contaminated systems and ecological and toxicological processes are needed in addition. However, the bioassays are valuable tools in the characterization of the toxic action of chemicals as pre-market toxicity testing or in the understanding of associated toxicity [Van der Oost et al., (2003)] or in monitoring the quality of surface waters by investigating integrated toxic effect potentials of the "cocktail" of substances present in the aquatic environment [Marinescu et al., (1997)]. If bioassays are not able to identify culprit chemicals which mainly contribute to toxic potential, combining them with chemical analysisis is extremely useful to obtain a complete image in hazard/risk assessment [Schuetzle & Lewtas (1986)].

In December 2000, European Union (EU) published new directives in the field of water which were recommended to all member states to assess the ecological situation of aquatic environment and to define their objectives and strategies with the aim to achieve a good quality of water until 2015. In addition, the directives also impose the determination of toxic substances concentrations, the elaboration of a list of all of these substances and their admitted concentrations based on biological criteria. The framework directives underline the importance of biological and ecological character to establish classic criteria of water quality involving a scientific cooperation between all of the member states of EU for standardization of these methods. This directive is revolutionary because it recognizes the importance of the biological and ecological criteria in determining water quality, requires the development of methods for

determining their ecological quality based on different benthonic invertebrates species, fish, etc. and involving scientific cooperation without borders among all EU member states to standardize these methods. Implementation of the Directive on a European scale is a challenge for monitoring the aquatic environment. The increasing complexity of environmental degradation requires an increase in the capacity of scientific approach, its monitoring and notification as early as possible risks.

In this context, environmental pollution and its detection is one of the encountered problems that benefit from a particular attention in our country and the world.

Our own objective concerns the detection of aquatic environment pollution in Romania and more particularly in the Danube basin, the biomonitoring of the pollution degree and the appreciation of the sanitary quality of food products of aquatic origin by applying original ecotoxicologic methods based on the use of viability and apoptosis biomarkers of nucleated red blood cells (RBCs).

The objective of this review is to summarize our results obtained for determining the toxicity of various pollutants based on nucleated erythrocyte apoptosis, by flow cytometry and complementary methods.

APOPTOSIS OF NUCLEATED ERYTHROCYTES - AS A TOOL FOR *IN VITRO* AQUATIC RISK ASSESSMENT

Cell death has been considered for a long time an uncontrollable, degenerative and catastrophic phenomenon, in body homeostasis as a response to cellular aggression. In recent years it was discovered, however, that all cells of multicellular organisms have the ability to activate a cell death program called *apoptosis* [Kerr et al., (1972); Raffray & Cohen (1997); Wyllie et al., (1980)] which changed the original idea and led to a revaluation of all biological aspects including immunology, development, carcinogenesis and more recently toxicology or ecotoxicology. Toxicologists (ecotoxicologists) have long time considered that the cells can be killed by a variety of toxic conditions, which, depending on the concentration, causing major cell damage or disruption of cellular environment, leading to necrosis (cell explosion). Now it is known that apoptosis is the major form of pathophysiological death (cell suicide) and necrosis occurs only in circumstances when aggression is caused by strong harmful substances. Apoptosis shows today a major interest in molecular toxicology, playing a central role in the action of many toxic substances, giving it an important role in their potential toxicological study.

Programmed cell death (PCD) of nucleated erythrocytes-a typical apoptotic phenomenon

The blood of all vertebrates, except mammals, contains lifelong (with rare exceptions) nucleated red blood cells, which is one of the universal features that distinguish mammals from non-mammalian.

Erythrocytes of lower vertebrates (fish, amphibians, reptiles, birds) are nucleated with an oval or ellipsoidal shape. Despite the presence of the nucleus, their specific function is reduced. The most important dimensional particularities are the diameter and volume. Normal erythrocyte has (in a ratio of about 2/3 of the cases), the average of 7,2μ as diameter. Mean erythrocyte thickness is about 2μ.

The erythrocytes of fish, amphibians, reptiles and birds are typically ellipsoidal, flat, biconcave shape caused by a bulged nucleus [Andrew (1965); Rowley & Ratcliffe (1988)]. In contrast, all adult mammalian blood erythrocytes are anucleate and they have a biconcave discocyt flattened shape. Their cytoskeleton is mainly a membrane skeleton containing proteins from spectrin and actin family, marginal a band of microtubules consisting of tubulin plus microtubule-associated proteins and intermediary desmin filaments [Fawcett & Witebsky (1964); Davies (1961); Cohen (1978); Cohen (1991)].

Though have the same function, the different types of vertebrate erythrocytes have neither the same size nor the same structure.

Vertebrate erythrocytes have a finite programmed life-span in the circulation which varies between species but is exceptionally constant with species: 900 days for frog, 700 for turtle, 120 for human, 115 days in dogs, 60 days in the rabbit and rat, 40 days the cat and mouse and 30 for chicken, for instance [Clark (1988)] as can be observed in figure 1.

The precision of such biological clock has intrigued many investigators who attempted to identify the signals that mark the RBCs to their removal from circulation by resident macrophages. The two modes of cell death (apoptosis and necrosis) differ fundamentally in their morphology, biochemistry and biological relevance. During the past years, active studies were focused on the mechanism of erythrocyte programmed cell death (PCD). We and others have shown that PCD of human erythrocyte and *Rana esculenta* nucleated red blood cells is an apoptotic phenomenon [Sakamoto et al., (1997); Bratosin et al., (2001); Bratosin et al., (2004); Bratosin et al., (2009)]. Despite their structural simplicity, the erythrocytes of lower vertebrates preserve nucleus and mitochondria, both the sensors of the PCD machinery to develop an apoptosis phenomenon. In our study, batracian *Rana esculenta* erythrocytes cell death induced by either calcium influx, or staurosporine, involves typical apoptotic phenotype. Our data showed: *i)* a drastic modification of the cell morphology with loss of the ellipsoidal form revealed by phase contrast microscopy and scanning electron microscopy (Fig. 2); *ii)* an exposure of the phosphatidylserine residues in the outer leaflet of the cell membrane (Fig. 3); *iii)* a caspase-3–like activity (Fig. 3); *iv)* a mitochondrial membrane potential ($\Delta\Psi m$) loss; and *v)* a condensation and nuclear fragmentation (Fig. 4). Erythrocyte chromatin condensation and fragmentation are prevented by caspase and calpain peptide inhibitors. These inhibitors also prevent protease activation-mediated $\Delta\Psi m$ loss supporting the idea that mitochondria are a central sensor for *Rana* erythrocytes cell death.

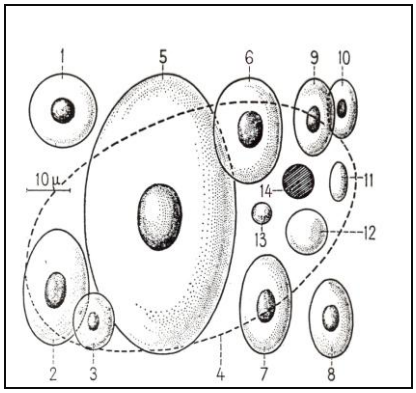

Figure 1. Various types of vertebrate erythrocytes. 1-Petromyzon marinus; 2-Raja; 3-Pleuronectes solea; 4-Amphiuma; 5-Proteus anguineus; 6-Rana; 7-Testudo graeca; 8- Lacerta; 9-Struthio camelus; 10- Gallus gallus; 11- Lama; 12- Elephas; 13- Capra; 14- Homo [Clark (1988)].

Our observations revealed the conservation of the programmed cell death machinery in erythrocytes across kingdom. Fish red blood cells, playing a central role in the physiology of respiration, are directly exposed to pollutants absorbed by different routes. These cells can be easily obtained and represent an outstanding model to study xenobiotic-induced damage to different cellular compartments. Little is known about the effect of environmental toxicants on apoptosis induction [Piechotta et al., (1999); Knopperld et al., (2005)].

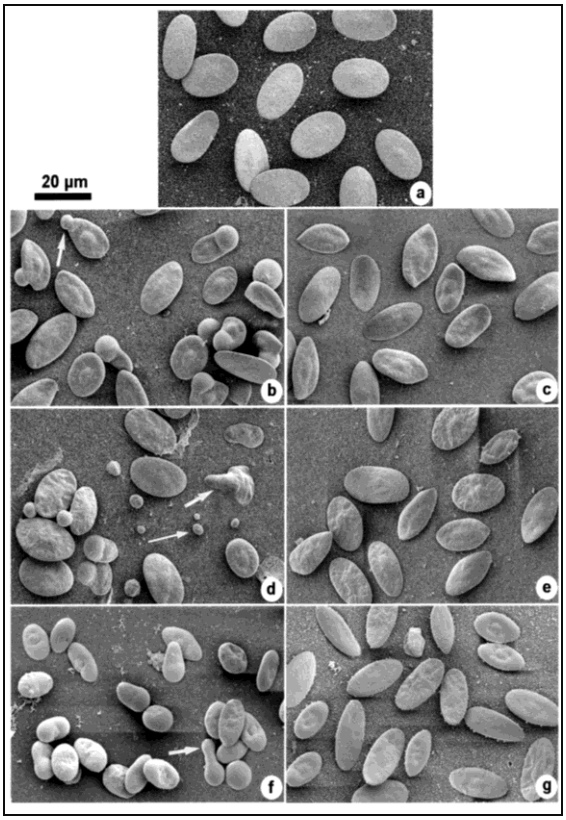

Fig. 2. **A**-Rana esculenta erythrocyte cell death. (**1**) Freshly isolated erythrocytes were incubated at 37°C for 6 hours in HEPES buffer pH 7.4 containing calcium chloride 2.5 mM and various concentrations of calcium ionophore A 23187. (**2)** Freshly isolated erythrocytes were incubated either in PBS buffer pH 7.4 in the absence (-□-) and presence (-●-) of staurosporine (10 µM) or in HEPES buffer pH 7.4 containing 2.5 mM calcium chloride in the absence (-■-) or presence (-○-) of calcium ionophore A 23187 (0,025 µM) or of staurosporine 10 µM (-▲-). Values indicate the percentages of erythrocytes remaining in the culture at the indicated time points [Bratosin et al., (2004)].

Methodology used to analyze in vitro toxicity (ecotoxicity) of pollutants.

Apoptosis is the result of the action of all toxic substances on the cells which consequently develops a characteristic morphology and specific biomarkers for quantifying cell death *in vivo* and *in vitro*. Flow cytometry (FCM) is certainly the most efficient methods for the study and quantification of apoptosis [Dive et al., (1992); Telford et al., (1994); van Engeland et al., (1998)].

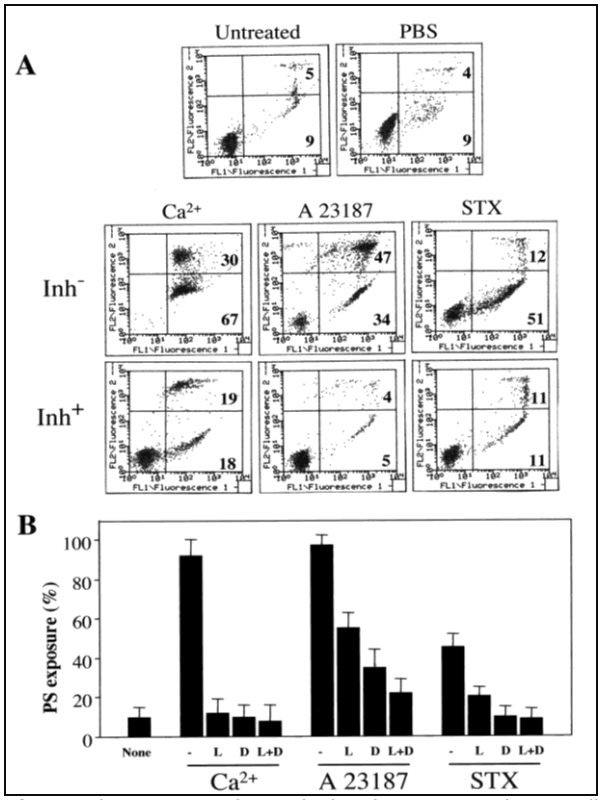

Fig. 3. Flow cytometric analysis of *Rana esculenta* cell death. Annexin V-FITC (FL1) and propidium iodide (FL2) were used to assess phosphatidylserine exposure and cell membrane integrity. Untreated/Freshly isolated (Untreated) erythrocytes or incubated at 37°C for 24 hours either in PBS (PBS) or HEPES buffer containing calcium chloride 2.5 mM in the absence (Calcium) or presence of ionophore A 23187 (0.025 µM). Cells were also treated with staurosporine (10 µM) (Staurosporine). Erythrocytes pretreated for 30 min in the absence (Inh^-) or presence (Inh^+) of a mixture of Ac-DEVD-cmk and leupeptin (200 µM each) in solution in DMEM culture medium,respectively. Cells were analyzed by flow cytometry. LL quadrant: viable cells (annexin V and propidium iodide negative cells); LR quadrant: apoptotic cells (annexin V positive and propidium iodide negative cells); UR quadrant: dead cells (annexin V and propidium iodide positive cells). Numbering refers to the cell percentage of each population. Number of counted cells: 10 000 [Bratosin et al., (2004)].

Flow cytometry is an analytical tool widely used to obtain detailed information on bioprocesses, using light-scattering, fluorescence and absorbance measurements. In flow cytometry, single cells or particles in a fluid stream pass through a laser beam and their absorption, scattering, and/or fluorescence can be monitored for each individual cell. The forward-scattered light provides information on the size of cells, sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology and the both can be detected without further manipulation. These data can be correlated with other different cell characteristics and cell components. In addition, flow cytometry has the advantage of being a statistical method, 10,000 or more cells can be analyzed in a very short time (a few minutes), which allows analysis of a large number of samples.

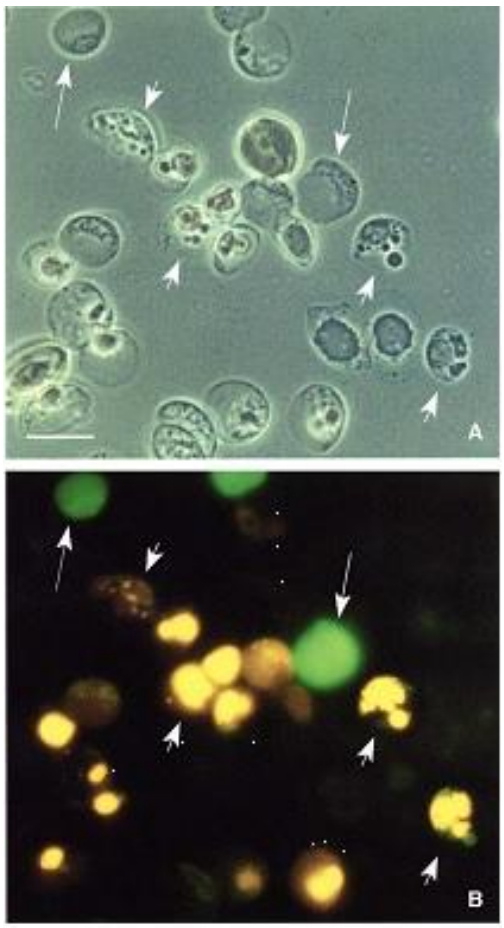

Figure 4. Changes in nuclear morphology of Rana esculenta erythrocytes. Phase contrast **(A)** and fluorescence **(B)** micrographs of Rana esculenta erythrocytes incubated (37°C for 6 h, then 20°C for 24h) in HEPES buffer pH 7.4 containing 2.5 mM calcium chloride. Erythrocytes were double-labeled with PhiPhiLux G_1D_2 (in green) and propidium iodide (in red). Yellow color corresponds to apoptotic cells. Short arrows indicate chromatin condensation and nucleus fragmentation. Long arrows are caspase positive cells. Bar: 30 μm [Bratosin et al., (2004)].

To assess aquatic pollution degree or for assessing cytotoxicity or ecotoxicity of pollutants (heavy metals, nanoparticles, etc.) we developed a new experimental cell system based on the use of nucleated red blood cells from fishes and batrachians which are directly exposed to pollutants or to nanoparticles absorbed by different ways. To evaluate cell-pollutant interaction, nucleated RBCs were exposed to different concentrations of pollutants or nanocomposites (nanoparticles) and analyzed by flow cytometry, after different times of incubation endpoints (6 to 24h) for morphological changes (FSC/SSC), apoptosis/necrosis analysis (FITC-annexin-V labeling/PI) and cellular viability (calcein-AM method). Technique designed to identify, quantitate and characterize apoptosis flow cytometry remains the methodology of choice to study the apoptotic phenomenon, especially when mixed cell populations need to be analyzed and differentiate more than one parameter simultaneously [Dive et al., (1992); Darzynkiewicz et al., (1997); Vermes et al., (2000)].

Detection of altered morphology by light scattering flow cytometry in the mode FSC/SSC and microscopy

Multiparametric flow cytometric analysis which discriminates and quantifies viable, apoptotic and necrotic cells via measurement of altered morphology by light scattering flow cytometry in the mode FSC/SSC provides informations about cell size and structure. The intensity of light scattered in a forward direction (FSC) correlates with 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. The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, a rounded appearance of the cell, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. During apoptosis, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents [Darzynkiewicz et al., (1997)]. Analysis of morphological changes by scattered light flow cytometry in the mode FSC/SSC is a very rapid and sensible method.

For exemplification, as shown in figure 5, flow cytometric analysis announce significant morphological changes of nucleated erythrocytes incubated for 24 h in saline supernatants of different nanomaterials (porphyrin base or metalloporphyrin

from P1-P8) compared to nucleated RBCs incubated only in saline isotonic solution (T24h).

In fact, the XGeoMean values (cell side scatter) vary from 168 (P6) to 268 for P3 as compared to the statistical value of normal RBCs, i.e. 182±6. In the same way, the YGeoMean 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±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. 6).

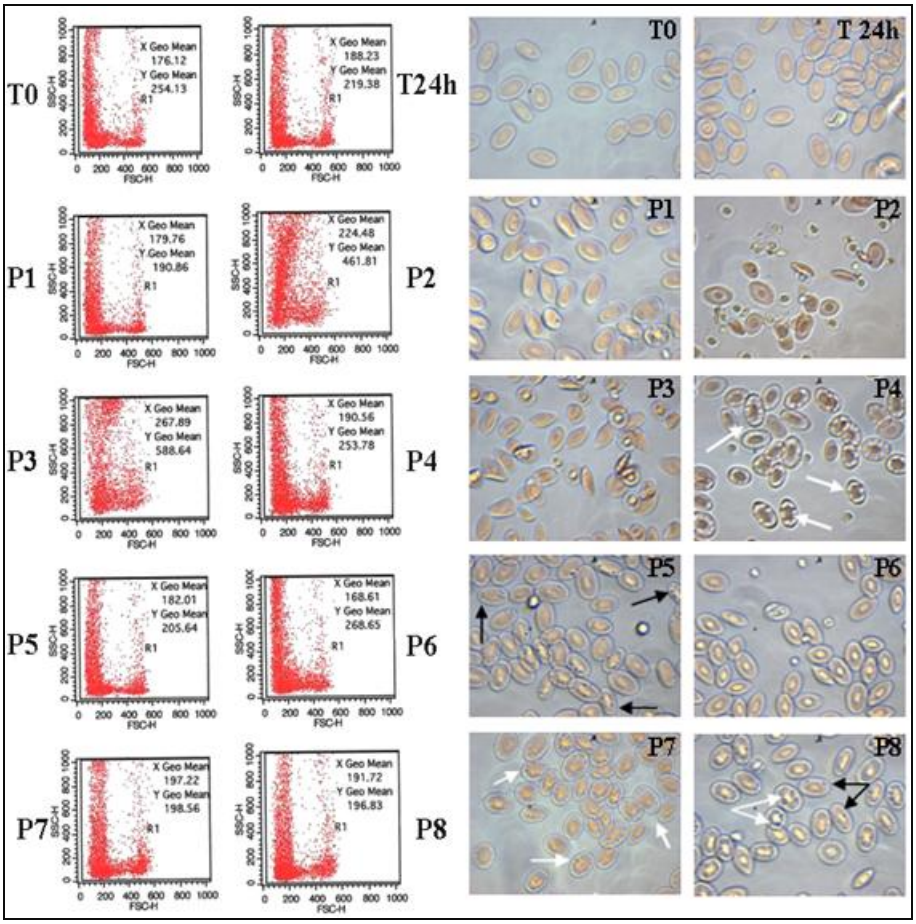

Fig. 5. Comparative morphological shape changes analyses by flow cytometry (A) and optical microscopy (B) of normal nucleated erythrocytes (To and T24h) and exposed to nanomaterials (P1 to P8) at 0,008 g/ml. Nanoparticles based on porphyrins and their hybrids: *meso*-tetra-tolylporphyrin (P1); hybrid silica- meso-tetratolylporphyrin by sol-gel reaction in two steps acid-base catalysis HCl:TEOS = 0.02:1; NH_3:TEOS=0.0142:1 (P2); Zn(II)-meso-tetrakis(4-pyridyl)porphyrin (P3); hybrid silica- Zn(II)-meso-tetrakis(4-pyridyl)porphyrin, acid catalysis HCl:TEOS=0.01:1 (P4); hybrid silica- Zn(II)-meso-tetrakis(4-pyridyl) porphyrin, acid/base catalysis HCl:TEOS=0.01:1; NH_3:TEOS=0.015:1 (P5); *meso*-tetra(3,4-dimethoxy-phenyl)porphyrin (P6); hybrid silica- *meso*-tetra(3,4-dimethoxy-phenyl)porphyrin acid catalysis (P7); hybrid silica- *meso*-tetra(3,4-dimethoxy-phenyl) porphyrin acid/base catalysis (P8) were synthesized according to the previous reported literature [Fagadar-Cosma et al., (2007); Fagadar-Cosma et al., (2009); Enache et al., (2010); Fagadar-Cosma et al., (2009)].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) [Bratosin et al., (2011a)].

Study of erythrocytes death by annexin-V-FITC and propidium iodide double -labelling

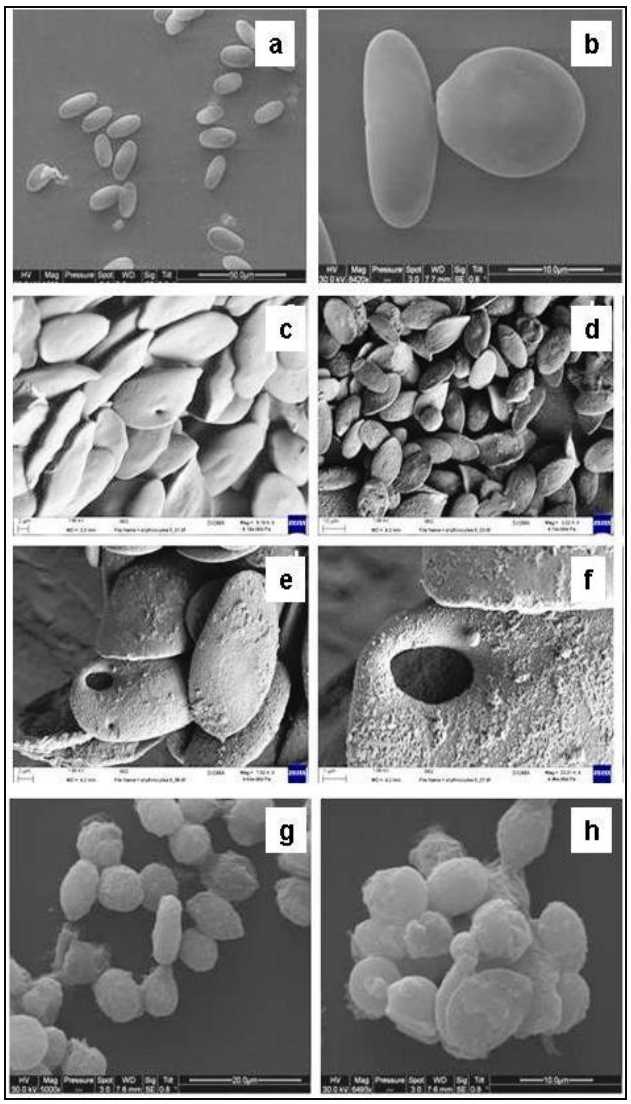

Fig. 6. Scanning electron microscopic analysis of normal nucleated erythrocytes (a and b) of *Rana sp.,* exposed to the action of sample P1 (meso-tetra-tolyl-porphyrin) at 0.008 g/ml (c-f) and to the sample P3 (Zn(II)-meso-tetra-piridil-porphyrin) at 0.008 g/ml(g, h). The results presented are representative experiments [Bratosin et al., (2011 b)].

Another popular technique is the detection of phosphatidyl serine (PS) normaly restricted to the inner surface of plasma membrane bilayer. To investigate the mode of cell death induced by diferents pollutant, we applied simultaneous staining of erythrocytes with annexin-V-FITC and propidium iodide (PI). 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.

Normal and incubated erythrocytes were analyzed by flow cytometry for phosphatidylserine (PS) exposure (annexin-V labelling) and membrane permeabilization (PI-labelling).

Figure 7 shows comparative flow cytometric analyses of normal (N) and incubated erythrocytes with P8 sample of porphyrins. We can see that porphyrin base or porphyrin-nanomaterials has *in vitro* serious deleterious effect on nucleated erythrocytes in a dose-dependent, allowing calculating EC50. The number of living cells (annexin$^-$/PI$^-$) decreased drastically from 96% (normal erythrocytes) to 23% for P3. 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 (or others pollutants) was further increased and the information could be potentially useful for the development of low cost and rapid ecotoxicity assays. Erythrocyte nucleated apoptosis can be an efficient ecotoxicological biomarker providing significant information of environmental stress.

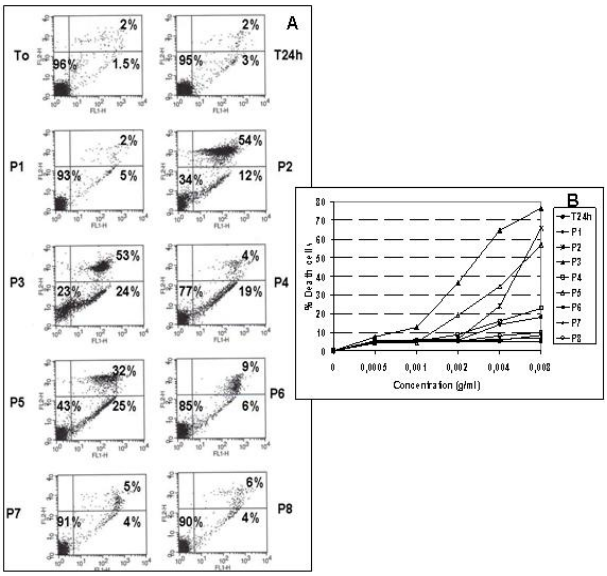

Figure 7. A**:** Comparative flow cytometric quadrant analysis of Annexin-V-FITC/propidium iodide double-stained of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml nanomaterials (P1 to P8). Abscissae: log scale green fluorescence intensity of annexine-V-FITC (FL-1). Ordinates: log 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_{50} 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 7A. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed [Bratosin et al., (2011a)].

Cell viability measured with Calcein-AM assay

For the measurement of red blood cells viability, we recently devised a new flow cytometric assay 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. 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 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 8, 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 EC50 (Fig. 9).

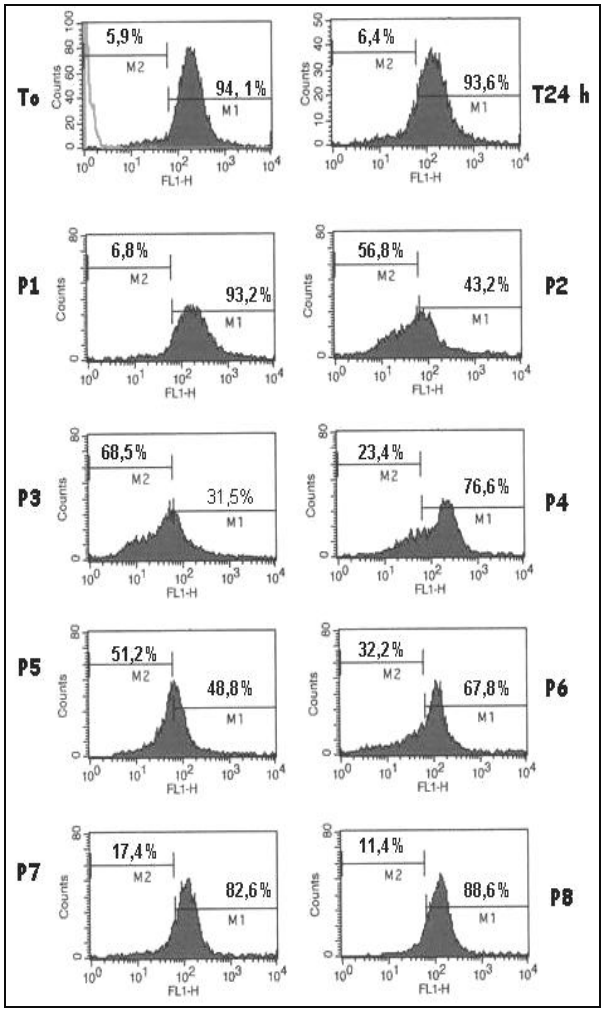

Fig. 8. Comparative flow cytometric histogram analysis of calcein-AM cell viability of normal nucleated erythrocytes (To and T24h) and exposed at 0,008 g/ml porphyrin-nanomaterials (P1 to P8). M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Abscissae: log scale green fluorescence intensity of calceine (FL1). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed [Bratosin et al. (2011a)].

IN VIVO DETECTION OF TOXICITY AND THE SAFETY OF FRESH FISH PRODUCTS DETERMINATION

Biosphere pollution due to evolution of technological civilization has not only consequences for the sustainability of plant and animal species, it compromises the future of humanity by acting on natural resources, in particular on compliance of agricultural productivity in various ecosystems.

Dispersion of toxic substances in the natural environment leads to more dangerous contamination of human food chains in which we are integrating in the top of the ecological pyramid [Lagadic, (1997)]. Discharge of all waste resulting from human activities in the environment exposes us to a boomerang effect through food chains.

Pollution of food or more specifically, human contamination of consumption is one of the most worrying environmental problems. Pesticides [Muirhead-Thomson (1971); Fournier (1974)], heavy metals [Holden (1973)] use of antibiotics and even hormones contribute substantially to the contamination of food chains. Food additives, colorings, stabilizers, emulsifiers not justify its presence and should be banned. In addition, aquatic food products are also becoming contaminated more. Food additives, colorings, stabilizers, emulsifiers not justify its presence and should be banned. In addition, aquatic food products are also becoming contaminated more. World Ocean is at present almost its entire polluted area [Peres (1976)] with various hydrocarbons contained in crude or degradation products resulting after action of biogeochemical actors. Only oil

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contamination, for example, is extremely high, about 5 million tons of crude oil contaminating ocean waters. This has directly consequences on phytoplankton, algae and the zooplankton. On the marine animals, contamination of fish and other marine animals has direct consequences for marine or oceanic origin foods. Scallops or fish collected from the neighboring coast industrialized regions contain 5 to 10 times more oil than those from unpolluted areas. "Taste of oil", even if not a direct toxic fish, leads to economic loss and prevent them from commercialization [Ramade (2000)].

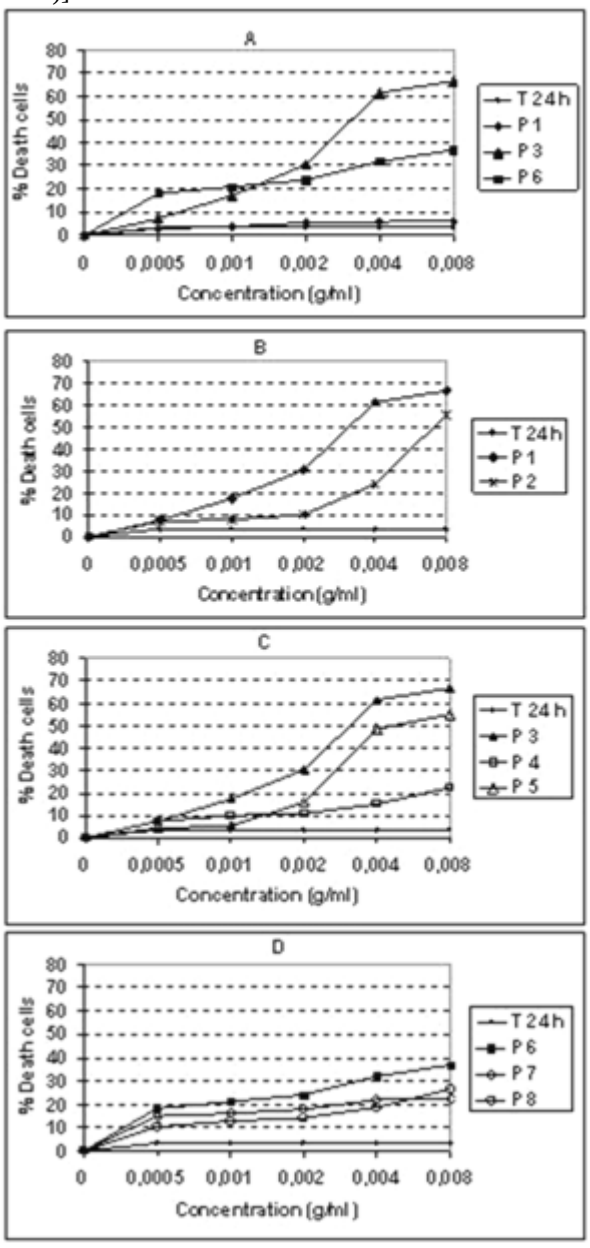

Fig. 9. Curves dose-response for the calcule of EC_{50}. Abscissae: concentration of porphyrin- nanomaterials. Ordinates: % of death cells coresponding of M2 region from flow histograms presented in figure 8. Results presented are from one representative experiment of three performed [Bratosin et al., (2011a)].

In present, the human society 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. The researchers are trying new biological tests for identification of new, sensitive biomarkers able to measure immediate and later effects of different substances on the aquatic environment, in general, to the pisciculture food and on the human health especially [Van der Oost et al., (2003)]. The bioassays probably respond the best to the current need to determine sanogenesis origin products used as food fish.

In this general context, we aimed to test whether *in vivo* apoptosis of nucleated fish red blood cells can be simultaneously a sanogenesis test, assessing the conditions under which a fish product was obtained. To verify our hypothesis we measured by flow cytometry and microscopy *ex vivo* nucleated red blood cells from the fish undergoing the sub lethal heavy metal poisoning in laboratory, at 24 h after acute intoxication with 0,01mM Hg as presented for illustration in figure 10.

BIOMONITORING:

A major characteristic of pollution from human origin consists of voluntary or involuntary dispersion of pollutants (pesticides, hydrocarbons, nanoparticles, etc.) or chemical elements capable to contaminate various compartments of the biosphere. In present there are no ecosystems that are free from traces of human activity because even the areas free from any inference are contaminated with pollutants brought by the movements of air masses or marine and oceanic currents.

The biggest problem is related to determining the maximum tolerated dose for each pollutant and especially the combination of several micro-pollutants, the multiplicity of pollutants or their synergism, where lapses in current knowledge are numerous and important. The contamination of the aquatic environment is traditionally presented in terms of concentrations of chemical contaminants in the environment. Traditionally, water quality monitoring actions have focused on physical and chemical measurements which have the consequence the fact that the effects produced by combinations of substances remain unknown [Tonkes et al., (1998)]. However, these concentrations do not give an estimate of the adverse effects on organisms, which involves almost mandatory today, the use of biological criteria obtained by bioassays [Chapman & Long, (1983)]. Analysis results of these bioassays or toxicity thresholds have determined potential candidates from aquatic environment of accidental or chronic manner, having "biological quality" of environment and its potential toxicity [Quiniou et al., (2005)]. Consequently, biological monitoring, or biomonitoring, is a very important tool to assess the condition of aquatic ecosystems, but the best indication of pollution

is a mix of chemical and biological information [Cairns (1995)].

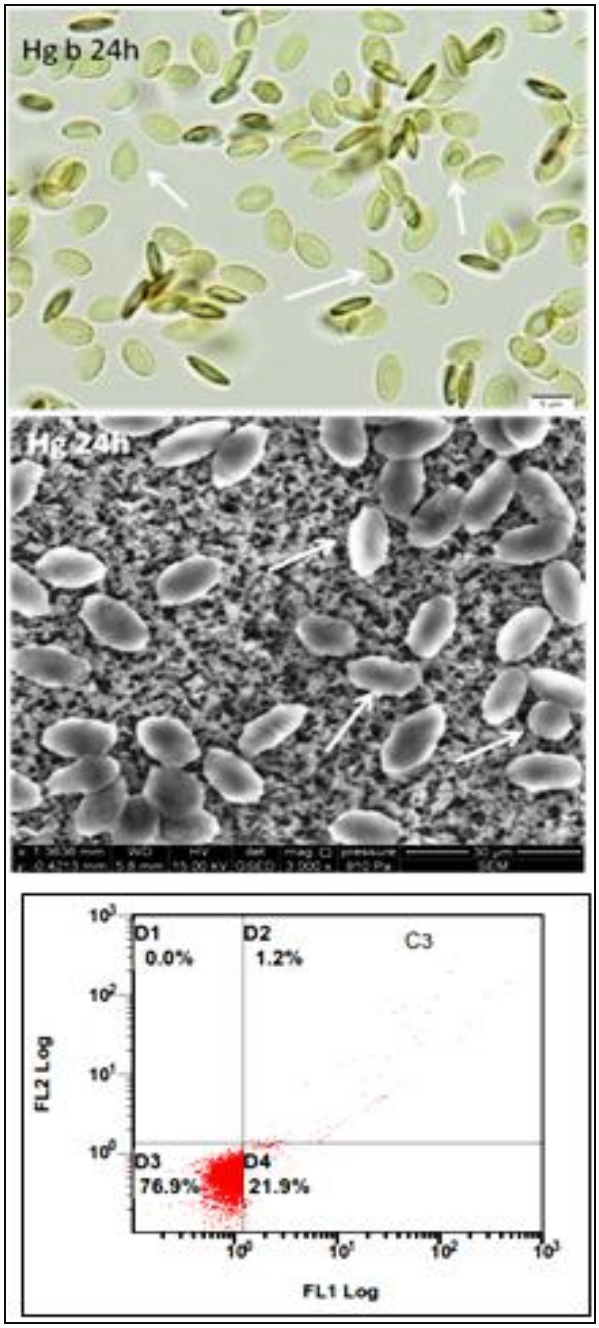

Fig. 10. *Ex vivo* analysis of *Carassius auratus* erythrocytes at 24h after acute intoxication with 0,01mM Hg by optical **(A)** and scanning microscopy for morphological changes **(B); (C):** quadrant analysis technique to identify the viable, apoptosis and necrosis cells by double staining with annexin-V-FITC (FL1) and propidium Iodide (FL2). % of viable cells:76.9% (low left quadrant); % of apoptotic cells: 21.9 (low right quadrant) [Covaci et al., (2013)].

In this sense, in order to verify the new bioassay developed to determine *in vitro* toxicity and ecotoxicity by flow cytometry based on apoptosis of nucleated red blood cells, we tested *in vitro* many different samples of water with different degrees of pollution from the affluent River of Danube Basin, the Danube River and the Danube Delta, using nucleated fish or amphibians erythrocytes exposed for different times. Three demonstrative results obtained using erythrocytes of *Carassius auratus* after 3 hours incubation *in vitro* are shown in figure 11. The percentage of apoptotic and necrotic cells was directly proportional to the degree of water pollution, ranging between 87% for P1, 61.95% for P2 and 99.9 for P3, compared to 23% for the control incubation sample, demonstrating validity of the test. Our preliminary results obtained in biomonitoring of Danube Basin pollution are really encouraging.

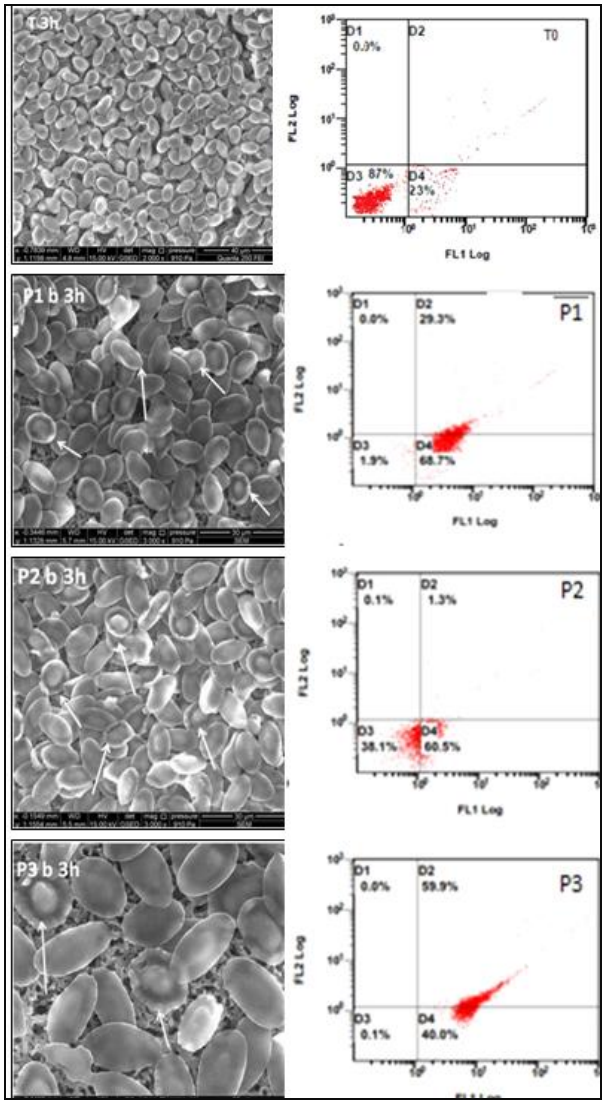

Fig. 11. Scanning microscopy and comparative flow cytometric analysis by quadrant technique to identify at the same time the viable, apoptotic and necrotic cells, after double staining with Annexin-V-FITC (FL1) and propidium iodide (FL2) of erythrocytes of *Carassius auratus* exposed *in vitro* 3 hours to the water samples (P1, P2, P3) with different degrees of pollution from Danube basin, compared to control sample T0. Abscissa: intensity of green fluorescence of Annexin-V-FITC (FL1) in logarithmic scale. Ordinate: logarithm of the red intensity of propidium iodide (FL2). Lower left quadrant: viable cells (Annexin - / PI -); Lower right quadrant: cells are in apoptosis (Annexin + / PI -); Top right quadrant: dead cells (Annexin + / PI +). The percentage of cells refers to the percentage of cells in each population. Number of cells analyzed 10,000.

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CONCLUSION:

Many biological techniques are now applied or developed to know the future and the impact of toxic pollutants. Their development is considerable and numerous recent applications are now possible. Still remains to develop other scientific advances to accomplish in this field still little investigated by researchers. Recent measures aims to better assess environmental risks pollutants or quality of aquatic systems. After the era of analytical chemistry, today, the use of bioindicators, species or groups of species bioaccumulating and sentinels which link bioindicators with biomarkers (molecular, biochemical, cellular, physiological or behavioral biomarkers) and the bioassays offers the possibility of a complete analysis for aquatic risk assessment. Our proposed bioassays which are based on the apoptosis phenomenon as induced biomarker by pollutants on fish or amphibians erythrocytes, evidenced by flow cytometry, could be rapid and very sensitive tests for in laboratory aquatic risk assessment and biomonitoring. In addition, it is possible comparing *in vitro* effects with that of *in vivo*, on the same cell type. Standardization and application of these tests will surely provide the opportunity of their use easily in ecotoxicological laboratories, biomonitoring of large river basins such as the Danube River Basin and will be also able deliver information on fish as a food product.

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