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DEVELOPMENT BY FLOW CYTOMETRY OF BIOASSAYS BASED ON *CHLORELLA* FOR ENVIRONMENTAL MONITORING

Constantin-Marian Petrescu [1*], Violeta Turcus [2], Ciprian-Valentin Mihali, Iulian Octavian Stana, Daniela Bratosin [2,3]

[1] Institute of Life Sciences, "Vasile Goldiș" Western University of Arad, Romania
[2] Faculty of Medicine, Department of Biology, "Vasile Goldiș" Western University of Arad, Romania
[3] National Institute for Biological Science Research and Development, 296 Spl. Independentei, 060031-Bucharest, Romania

ABSTRACT: 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. The increasing complexity of environmental degradation requires an increase in the capacity of scientific approach in monitoring and notification as early as possible risks.

Our own objective concerns the detection of aquatic environment pollution in Romania and particularly in the Danube basin. For assessing aquatic environment pollution degree or for assessing cytotoxicity or ecotoxicity of pollutants (heavy metals, nanoparticles, pesticides, etc.) we developed news experimental bioassays based on the use of viability and apoptosis biomarkers of *Chlorella* cells by flow cytometry.

Our proposed bioassays could be rapid and very sensitive tests for in laboratory aquatic risk assessment and biomonitoring.

Key words: bioassays, aquatic pollution, cytometry, biomonitoring

INTRODUCTION:

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 on European and global level, because these are related to another major objective which follows biodiversity protection. Eutrophication caused by global industrialization and anthropogenic impacts on ecosystem can lead to biological damage, an impact to long-term on the health of human population and perhaps even on human evolution as a species. 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.

Both marine microalgae and freshwater tests were designed using the flow cytometry detector and were applied to test wastewater, chemicals and water with sediment. Bioassays algae have been widely used to assess the potential impact of contaminants in freshwater and marine systems.

Our research focused primarily on identifying the effects of heavy metals, for determining risk concentrations to health and environment, through new cell investigation based mainly on flow cytometry techniques.

Investigation of the toxic effect of heavy metals on microalgae was performed by flow cytometry techniques and complementary methods, as optical and scanning electron microscopy. The main purpose of this analysis was to identify a sensitive manner method able to reveal pollutants such as heavy metals and which is subsequently converted into a biotest based on flow cytometry.

In this purpose we analyzed changes in morphology by flow cytometry, determination of the metabolic activity based on the level of activity of esterases with Calcein-AM (test for cell viability), the measurement of reactive oxygen species (ROS) and determination of the membrane potential using $DIOC_6$.

MATERIALS AND METHODS

Chemicals

3,3′-dihexyloxacarbocyanine-iodide (DiOC6) and 2′,7′-dichlorodihydrofluorescein (H_2DCF), products obtained from Molecular Probes (Interchim, Montluçon, France), and to study the influence of the heavy metals it was used $Al(NO_3)_2$ x 9 H_2O ,CdO, Zn $(CH_3COO)_2$ x 2 H_2O, $HgCl_2$, Pb $(CH_3COO)_2$ x 3 H_2O, Cu SO_4 x $5H_2O$ and Pentachlorophenol (PCP-C_6HCl_5O) provided by Sigma-Aldrich (St. Louis, MO, USA).

The experiments presented in this work were performed in cell culture plate wells provided by Corning (Corning, NY).

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, Algological Laboratory from their 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.

Flow cytometric analysis

Flow cytometric analyses were performed on Cytomic 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.

***Correspondence**:* Constantin-Marian Petrescu, Institute of Life Sciences, "Vasile Goldiș" Western University of Arad, Romania, marian.petrescu@yahoo.com

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 FSC/SSC mode 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.

Metabolic activity analysis based on the level of esterase activities determined with Calcein-AM (cell viability test)

The metabolic activity was measured using a specific substrate for the esterases activity, Calcein-AM (acetoxymethyl ester of fluorescein), reagent much more permeable through the cell wall to fluorescein diacetate (FDA), product been previously used in a method reported as sensitive and quick to assess the metabolic activity of phytoplankton (Jochem 1999; Prado *et al.,* 2009). Calcein-AM is a lipophilic substance, non-fluorescent, which falls freely into cells, where it is cleaved by esterases nonspecific, generating a fluorescent product (fluorescein), product retained by the cells with membrane intact. Fluorescein fluorescence conferred by the cell is proportional to enzyme activity and therefore inhibition of this activity by esterases, thereby expressing the level of metabolic activity.

The method applied by us was the method developed by Bratosin *et al.,* 2005.

The method proves correlated with other flow cytometry tests may form the basis for a bioassay by flow cytometry easily applied in ecotoxicity studies.

Measurement of reactive oxygen species (ROS)

The intracellular generation of reactive oxygen species (ROS) is produced by oxidative stress witch can be caused by exposure of cells to heavy metals. The generation of ROS can be monitored using dihydro-rhodamine 123 (DHR123) and dihydroethidium (EH) which is oxidized by free radicals, H_2O_2 or NO_3. An alternative to them is the measurement of 2 ', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) which is converted by an intracellular esterase to a nonfluorescent product 2', 7'-dichlorodihydrofluorescein (H2 DCF). When induced ROS formation within the cell, it is converted to H2 DCF 2 ', 7'-dichlorofluorescein (DCF), fluorescent product which can be quantified (Yoshida et al., 2004; Saison et al., 2010).

Oxidative stress in the cells of microalgae *Chlorella* was evaluated after exposure of cells to different concentrations of heavy metals, based on the determination by flow cytometry intracellular levels for radical anion superoxide ($O_2^{\bullet -}$) by using the fluorescent substance H_2DCFDA. Fluorescence produced in the cell was collected with detector FL1 (505-550).

Determination of membrane potential with DIOC6

Membrane potential and respiration rate of the *Chlorella fusca* cells were monitored using a potentiometric probe which may reveal the dynamics of changes in membrane potential between the inside and outside of the cell. Cell membrane depolarization can be detected easily lipophilic dyes such as DiBAC4(3) or $DIOC_6$(3) (Adler et al., 2007; Jamers et al., 2009). The intensity of fluorescence is influenced by the penetration and removal of the fluorescent dye by the cell membrane electrochemical potential respectively and the number of lipophilic binding sites within the cell. This type of dyes showing the distribution of dependency load is sensitive markers in assessing cell viability.

As probe was used $DIOC_6$ (3), dye-permeable through the cell membrane, lipophilic selectively to intracellular membrane (Gregori *et al.,* 2002). Green fluorescence emission of this compound, with a maximum at 516 nm was collected detector FL1 (505-550 nm).

Optical and scanning electron microscopic analysis

Direct light microscopic analysis was performed using an equipped with the Olympus BX 43 Olympus VC-30 and the room visualization software CellSens Dimension. ESEM analysis was 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 4^0C until examination. After washing, *Chlorella* cells 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). Order the examination was 1500 mag, 3000 mag, 6000 mag and examination time was between 10 to 30 minutes.

RESULTS AND DISCUSSION:

Flow cytometric analysis of cell morphology changes by light scattering measurements and microscopic techniques.

Cell viability estimated by measuring the absorption and scattering of light, known as the "direct analysis in FSC/SSC system (light scatter measurements) is particularly simple and sensitive. After intercepting the incident light, the cell emits a certain number of signals. Light scattered at an acute angle or shaft (FSC) can be correlated with cell size, enabling to distinguish a cell aggregates or cellular debris and assessing cell viability, dead cells because the light diffuses weaker in this direction. A right angle light scatter (SSC) allows the study of refractive cytoplasmic cell contents and cytoplasmic ratio nucleotides.

All morphology changes observed by flow cytometry in "dot-plot" FSC/SSC system, quantified by XGeoMean respectively YGeoMean were correlated

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with analysis by optical microscopy and scanning electron microscopy (ESEM).

The experiments were followed at 24h, 48h, 72h and 6 days to identify the optimal time period for incubation with serial dilutions of heavy metals in order to establish a protocol for the application to bioassay that we want to imagine by flow cytometry.

The most obvious and representative results were obtained at 6 days of incubation, which is why we will limit the presentation. Also, we present only the results of the action of Al and Cd on *Chlorella fusca*, which is representative of our goal. Incubation of the microalgae with Hg, Cu, Zn and Pb generated similar results (results not shown), which bring more scientific information.

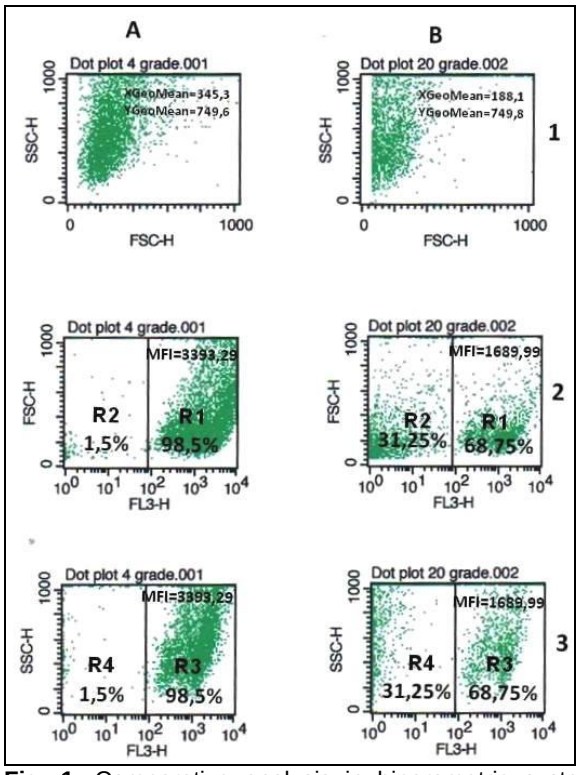

Fig. 1. Comparative analysis in biparametric system (dot plot) FSC/SSC (1) FL3/FSC (autofluorescence conferred by chlorophyll a / cell size) (2) and FL 3/SSC (autofluorescence conferred by chlorophyll a/ cell content) (3) for micro-algal culture, cultivated at incubation time T0 with heavy metal (a) and the control incubation represented by microalgae grown for 6 days with heavy metals (B).

Comparative analysis in biparametric system FSC / SSC (dot-plot FSC / SSC) of *Chlorella fusca* culture at time T0 to the control incubation for 6 days in the conditions of the experiments carried out with heavy metals, as seen in Figure 1, 1A and 1B show a significant change of morphology reflected the decrease in the value XGeoMean, from 345.3 to 188.1 without any disruption of cellular content. This change in cell size analysis is confirmed by ESEM, a very sensitive method, as can be seen from Figure 1, respectively (samples T0a, T0b and T0c compared with T6 days).

The comparison between dot-plots FL3/SSC (autofluorescence conferred by chlorophyll a/cell contents) for the two blanks (T0 and after T6 days of incubation conditions of the experiments without heavy metals) in Figure 1, A3 and B3, confirm exactly the percentage of non-chlorotic cells obtained from dot plots FL3/SSC analysis. It is noted at the same time, the reducing of MFI (Mean Fluorescence Intensity) value after 6 days of incubation in the conditions of the experiments carried out without heavy metals, from 3393.29 to control culture at time T0 to 1689.99 (almost half) , which means a reduction in the level of chlorophyll in the non-chlorotic cells.

Comparative analysis in biparametric system (dot plot) FSC/SSC for *Chlorella fusca* incubated for 6 days in the presence of various concentrations of aluminum (1-9), as it is shown in Figure 2, clearly demonstrate variations of the cell arrangement in the citogrames achieved. Thus, taking into consideration the values of XGeoMean (cell size) and YGeoMean (cellular content) of *Chlorella* samples incubated for 6 days with various Al concentrations in serial dilutions (1-9) compared to the control incubation without (T6 days) and witness the culture baseline (T0), values refer to dot-plot analysis in Figure 1, we see the following:

1. Compared to blank incubation T6 days, the XGeoMean (cell size) varies only for increased concentrations of Al, from 1mg /ml to 0.0625mg / ml. This variation is showed by reducing XGeoMean's from 419.12 to 166.83 for T6 incubantion blank to 1mg/ ml. concentration

2. The same decrease was observed for YGeoMean (cellular content) from 1 mg /ml to 0.0625mg /ml Al concentration, YGeoMean the decreasing from 800.31 for the T6 days control to 306.79.

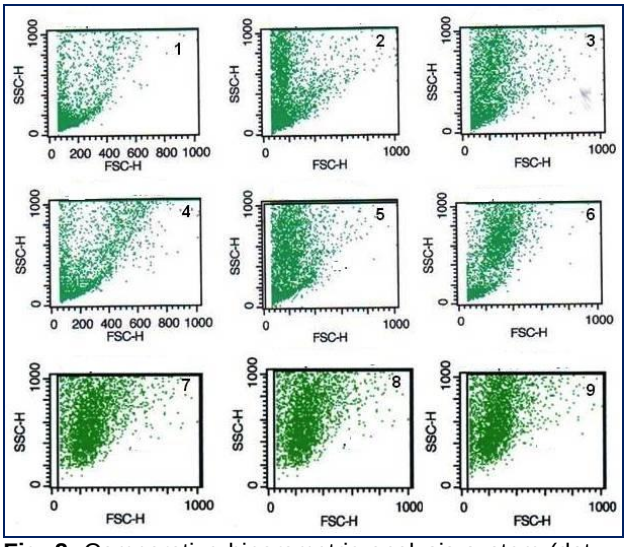

Fig. 2. Comparative biparametric analysis system (dot-plot) FSC/SSC of *Chlorella fusca* micro-algal culture incubated for 6 days in the presence of various concentrations of aluminum (1-9).

All *Chlorella fusca* samples incubated with various concentrations of Al were analyzed by optical microscopy, representative results being presented comparatively in Figure 4.

Also, the same samples analyzed by flow cytometry and observed by optical microscopy, were analyzed in detail for an additional scanning microscope ESEM.

For the control sample the *Chlorella fusca* culture at the start of experiments (T0), as shown in Figure 5 to different levels of detail, it can be observed the following:

1. The cells in the medium culture are spherical, very slightly elongated, with a relatively smooth surface, it in the form of colony forming units, with the cells embedded in an extracellular matrix.

2. *Chlorella* cells representing the control incubation begins to suffer slight changes in morphology (Fig. 6, T24h, T48h and T6 days) or the appearance of furrous.

3. In all cases control incubation T24h, T48h and T6 days, cell colonies remain in the extracellular matrix.

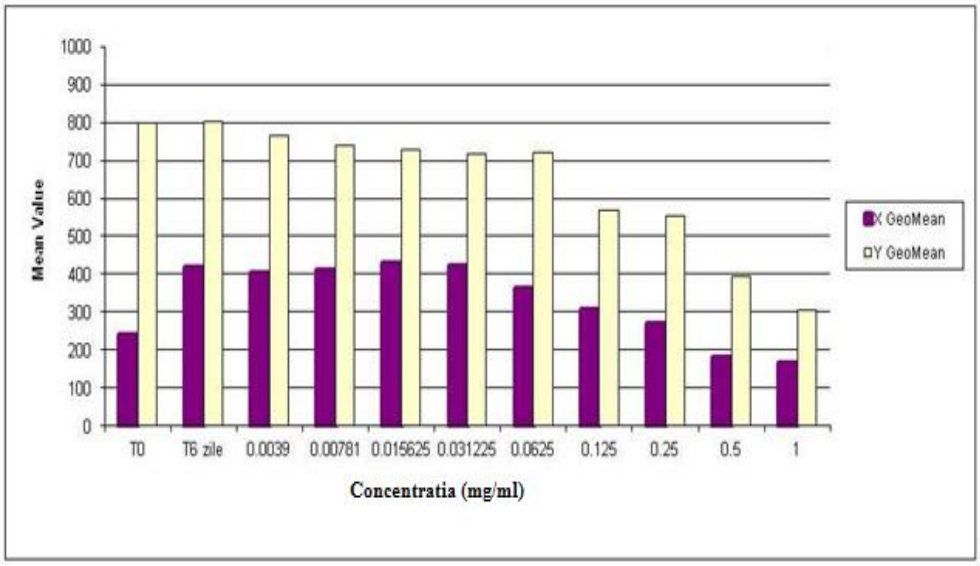

Fig. 3. XGeoMean comparative histogram (cell size) and YGeoMean (containing cells) of *Chlorella* samples incubated for 6 days with various Al concentrations in serial dilutions (1-9) compared to the control incubation without (T6 days) and base control of culture (T0).

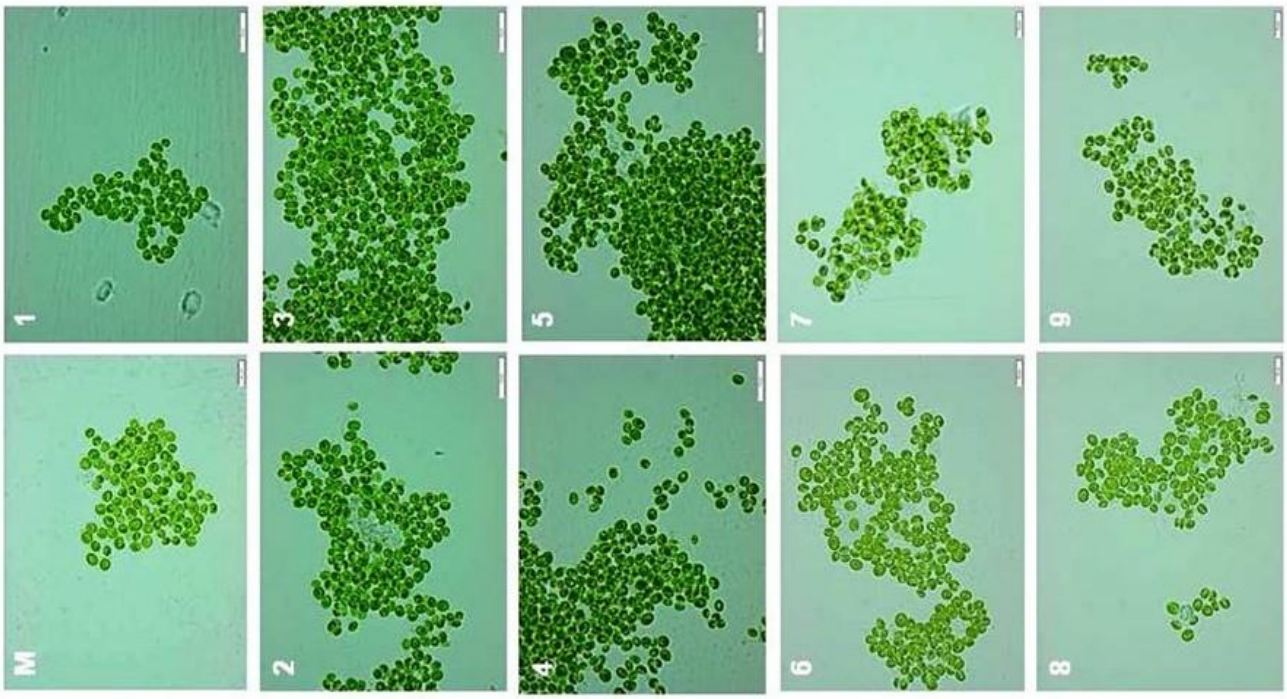

Fig. 4. Optical microscopy analysis for *Chlorella fusca* culture incubated for 6 days in the presence of various concentrations of aluminum (1-9), compared to the control incubation (M).

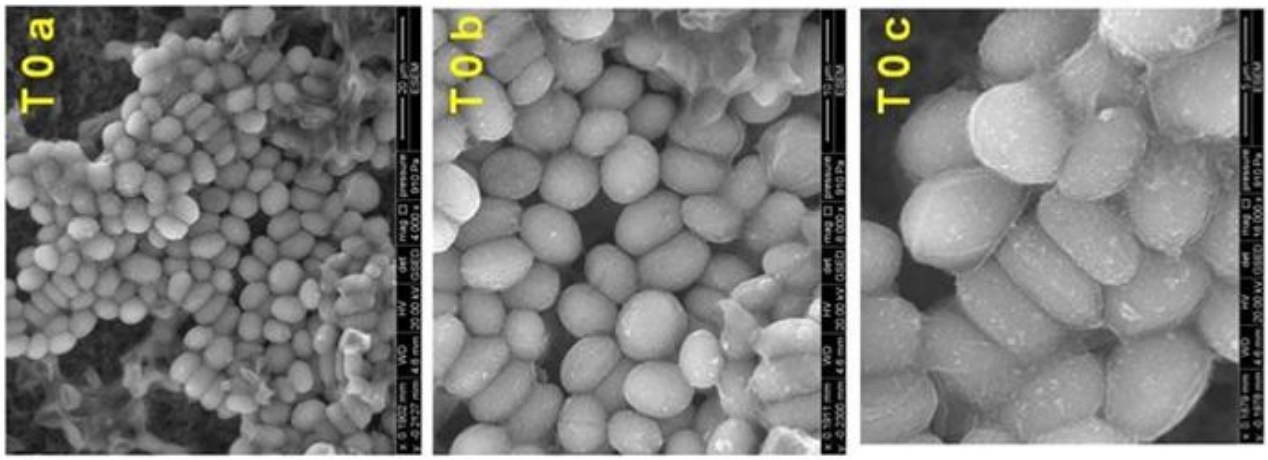

Fig. 5. Analysis by scanning electron microscopy (ESEM) of *Chlorella fusca* culture at the time T0. The results shown are representative of experiments performed.

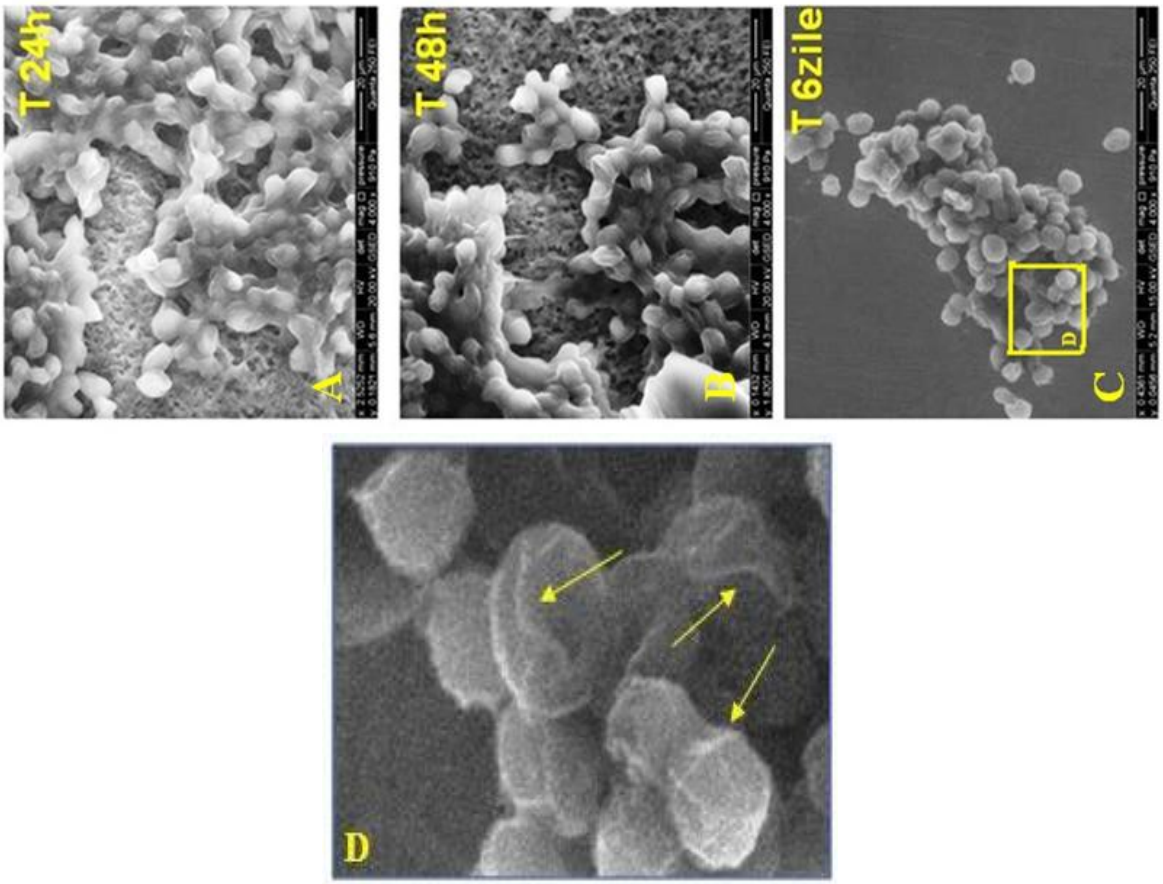

Fig. 6. Analysis by electron microscopy scanning of the *Chlorella fusca* control culture after 24h, 48h and 6 days of incubation with heavy metals. D: T6 days detailed morphology. Arrows indicate "furrows" on the surface of the cell wall. The results shown are representative of experiments performed.

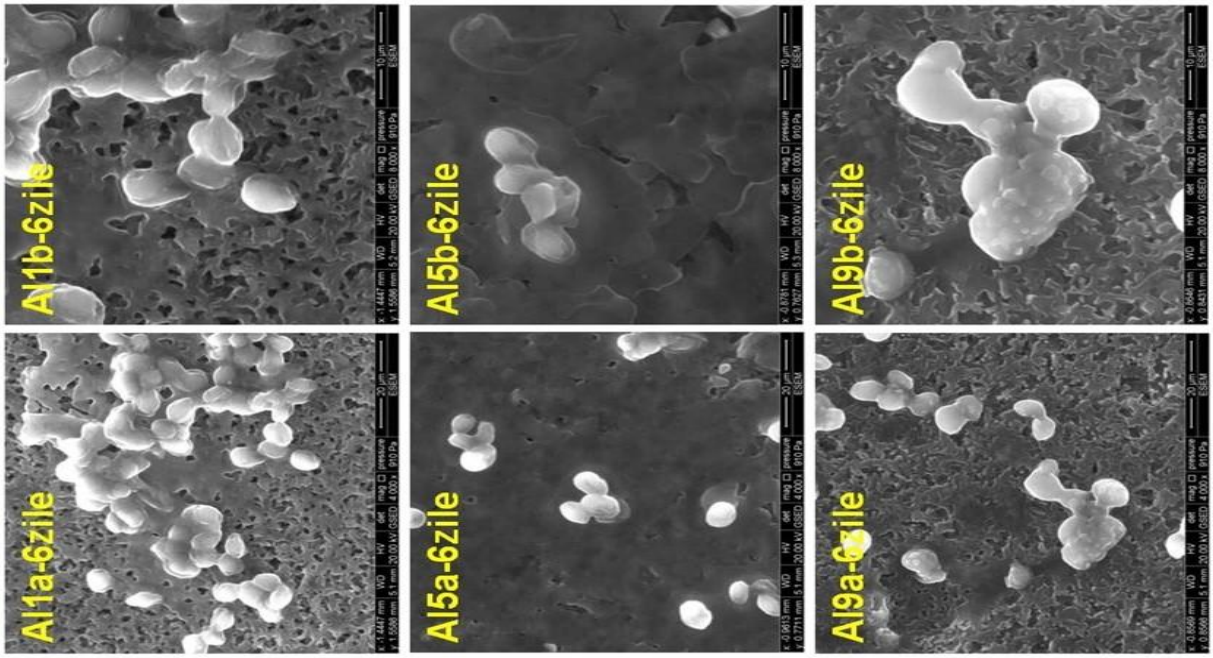

Fig. 7. Analysis by scanning electron microscope the morphology changes of the samples exposed *Chlorella fusca* at 1, 5 and 9 Al (serial dilution) concentrations for 6 days. The results shown are representative of experiments performed.

Due to Aluminium concentration for 6 days, as shown in Figure 7. the colonies are greatly diminished as size, and on the surface it could be observed "protuberances", the characteristic phenotype of cells in apoptosis (Figure 7 Al 9b).

If we analyze the microalgae *Chlorella fusca* incubated with different serial dilutions of Al (1-9) after 6 days of incubation by flow cytometry system FL3/FSC (autofluorescence conferred by chlorophyll a/cell size), and correlate the percentage of non-chlorotic cell from R1 and chlorotic cells from R2 with the same percentages for control in Figure 1 it can be seen that these percentages are proportional to the concentration of Al., ranging for healthy cells from R1 from 3.75% for the concentration of 1 mg/ml to 68.10 in the case of concentration of 0.0039 mg/ml, compared to 68.75% for the control incubation, which is approximately the same.

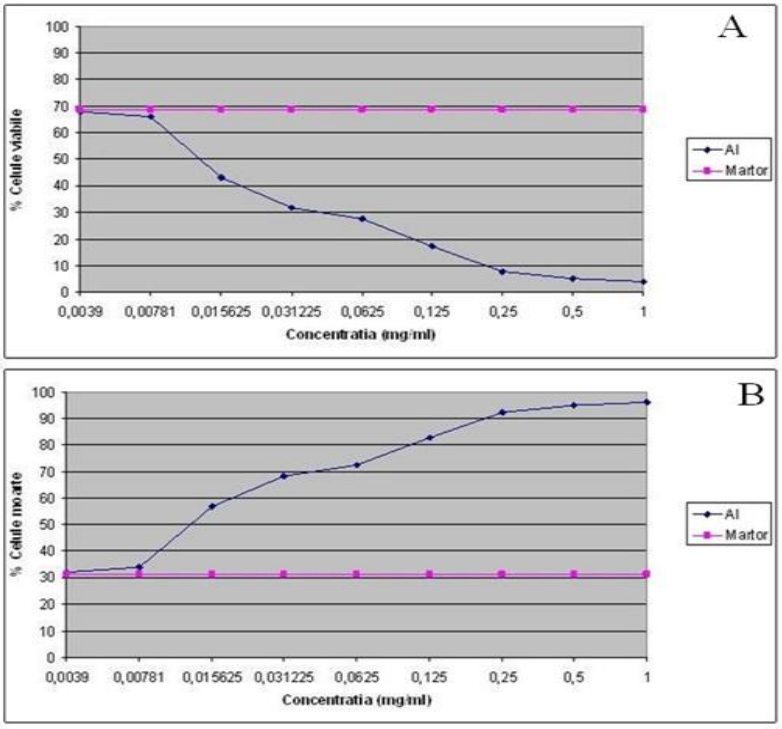

Fig. 9. Dose-response curves for the calculation of EC-50. Abscissa: concentration of Al. Ordinate: % of viable cells corresponding to the region R1, dead cells corresponding to R2 of dot-plots region shown in Figure 19. The results shown are representative of three experiments performed.

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For comparison, in the case of the microalgae *Chlorella fusca* incubation with different concentrations of cadmium have been used in the same concentrations in mg/ml (1-91 dilutions).

Comparative analysis system biparametric (dot plot) FSC/SSC after 6 days of incubation, as can be seen from Figure 10 and histogram comparative for XGeoMean (cell diameter) and YGeoMean (cell contents) of *Chlorella* cells incubated for 6 days with different concentrations of Cd serial dilutions (1-9) compared to the control incubation without Cd (T6 days) and Control group the culture T0 Figure 12 shows the induction of more dramatic changes. T6 to the control incubation days, the XGeoMean (cell size) varies only increased concentrations of Cd, from 1 mg/ml to 0.0625 mg/ml. This variation is manifested by reducing XGeoMean's witness from 419.12 to 179.9 days of incubation T6 concentration of 1 mg/ml.

The same decrease is observed for YGeoMean (cell content) from a Cd concentration of 1 mg/ml to 0.0625 mg/ml, reducing the YGeoMean site from 800.31 to 389.55 T6 for control incubation days. All samples of *Chlorella fusca* incubated with different concentrations of Cd were analyzed by optical microscopy, representative results being presented in a manner comparative Figure 12.

All samples were analyzed by flow cytometry and optical microscopy (Figure 12), and for added detail and scanning microscopy ESEM (Figure 13).

It appears that up to 7 Cd dilution, ie 0, 015 mg/ml, colonies of microalgae are much lower, with many non-chlorotic cells with alterations in cell wall and extracellular matrix.

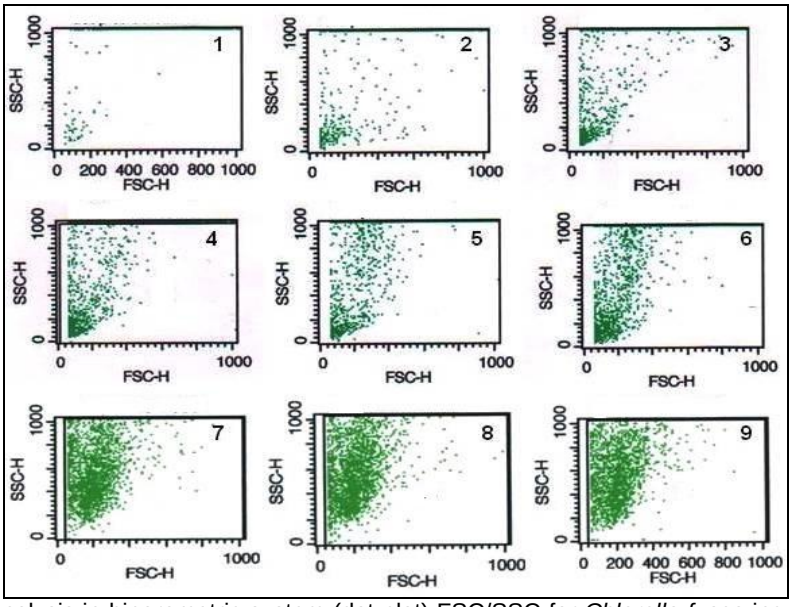

Fig. 10. Comparative analysis in biparametric system (dot-plot) FSC/SSC for *Chlorella fusca* incubated for 6 days in the presence of varying concentrations of cadmium (1-9).

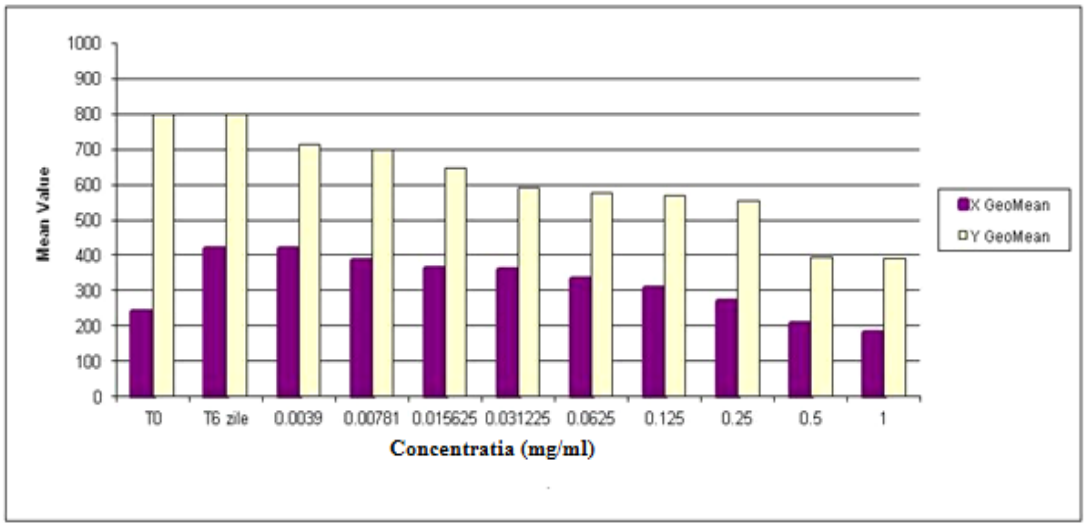

Fig. 11. XGeoMean comparative histogram (cell size) and YGeoMean (cell content) of *Chlorella* samples incubated for 6 days with different concentrations of Cd serial dilutions (1-9) compared to the control incubation without Al (T6 days) and the culture baseline (T0). The values refer to the dot-plot analysis of Figure 10. The results shown are representative of three experiments performed.

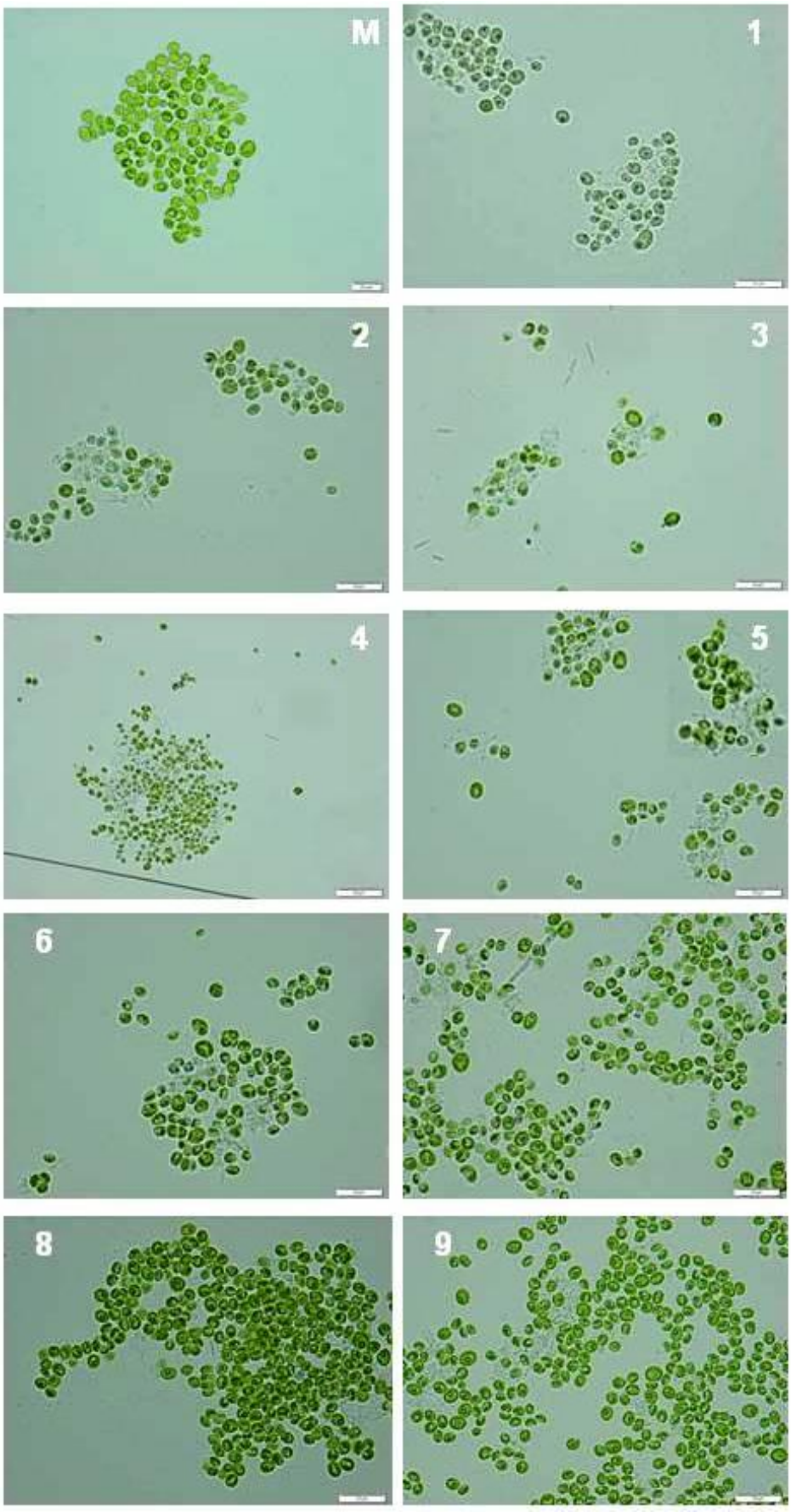

Fig. 12. Analysis by optical microscopy for the culture of *Chlorella fusca* incubation for 6 days in the presence of various concentrations of Cadmium (1-9), compared to the control incubation M.

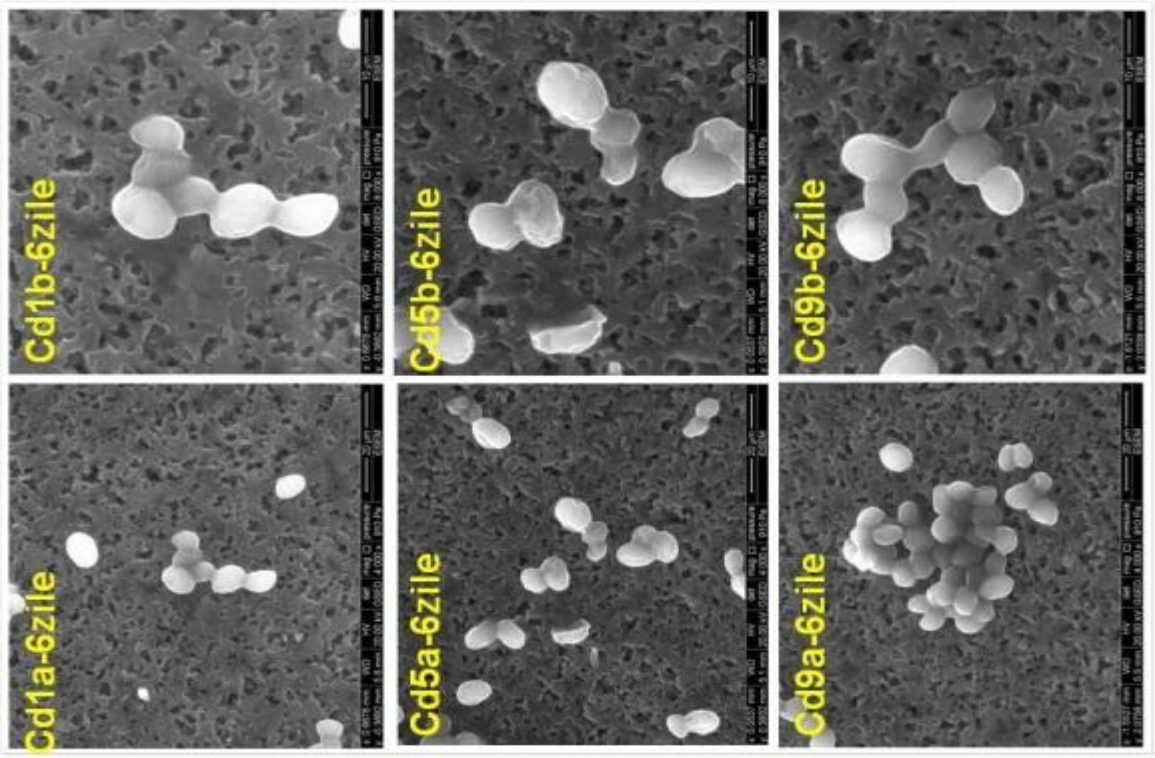

Fig. 13. Analysis by scanning electron microscopy of the morphology changes of the exposed *Chlorella fusca* sample concentrations of 1, 5 and 9 of Cd (serial dilution) for 6 days. The results shown are representative of experiments performed.

Analysis of *Chlorella fusca* microalgae incubated with different serial dilutions of Cd (1-9) after 6 days of incubation by flow cytometry system FL 3/FSC (chlorophyll autofluorescence conferred/cell size) as it is shown in Figure 14 , which are intended % of non-chlorotic cells (region R1) and % chlorotic cell of R2 compared to the same percentages for control in Figure 12, can lead to plotting dose-response curves to calculate the EC-50 where the abscissa is inscribed concentration of pollutant (in our case Cd) and the ordinate % of living cells, healthy, corresponding to the region R1, or cell chlorotic corresponding to the region R2 of the dot-plot sites shown in Figure 14.

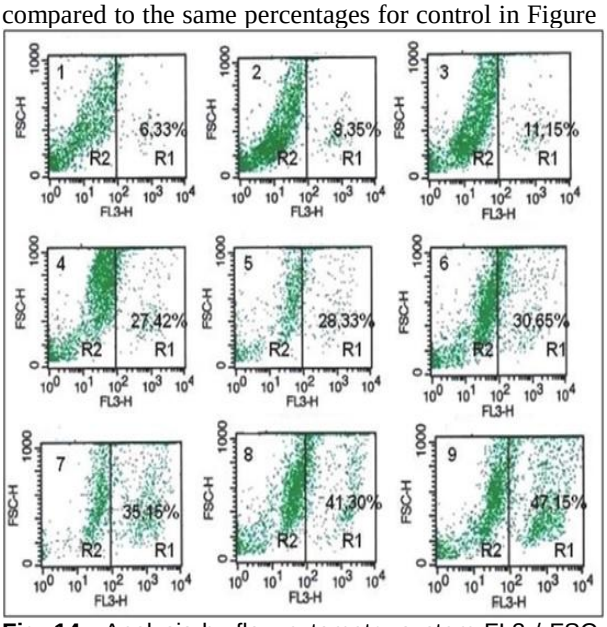

Fig. 14. Analysis by flow cytometry system FL3 / FSC for determining changes in the morphology of the microalgae *Chlorella fusca* of different incubated with serial dilutions (1-9) by the Cd after 6 days of incubation. (Analysis performed on a FACScan flow cytometer, Becton Dickinson)

Analysis by flow cytometry of microalgae *Chlorella* incubated for 6 days in the presence of various dilutions of heavy metals showed a perfect correlation and highlight morphological changes observed by flow cytometry and complementary techniques of investigation, so as were presented.

Metabolic activity analysis based on the level of esterase activities determined with calcein-AM (cell viability test)

The metabolic activity measured using specific substrate for the esterases activity, Calcein-AM (acetoxymethyl ester of fluorescein) was correlated with other flow cytometry tests may form the basis for a bioassay by flow cytometry easily applied in ecotoxicity studies

We could observe that pentachlorophenol has a high toxicity on the algal colonies, and they were very low and many cells chlorotic, and even some solitary algae in the extracellular matrix, proving information that PCP has a strong influence on photosynthesis and cellular metabolism. These changes were observed through flow cytometry analysis in FL 3/FSC system.

Morphological changes observed by scanning microscopy ESEM at various concentrations of the PCP and different incubation times, lead to appearance of a particular phenotype at a concentration of 0.079 g/L (0.3 mM PCP/L), where the cells have the appearance of "boiling" cell, phenotype characteristic

of eukaryotic animal cells in apoptosis, phenotype described by us under the influence of heavy metals, olso find in the scientific literature but also in a concentration of 100 mg/L-1 Cr (III).

The metabolic activity of the *Chlorella* cells with Calcein-AM is deeply affected, is inhibited at 24 hours and exhibiting a substantial increase to 48 hours, in particular for dilutions 5 and 6, is maximal after 6 days of incubation when the morphological changes were the deeper

Measurement of reactive oxygen species (ROS)

Oxidative stress in the cells of microalgae *Chlorella* was evaluated after exposure of cells to different concentrations of heavy metals, based on the determination by flow cytometry intracellular levels for radical anion superoxide ($O_2^{\bullet -}$) by using the fluorescent substance H_2DCFDA, fluorescence produced in the cell being collected with detector FL1.

According to the scheme on Figure 15, the calculation of ROS positive cells was done applying the dials, the biparametric system FL3/FL1, where FL3 is chlorophyll autofluorescence conferred by the green fluorescence and expressed FL1 oxidative stress.

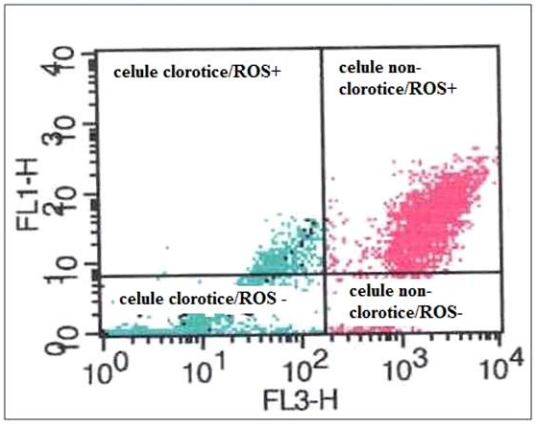

Fig. 15. Analysis by flow cytometry in biparametric system FL3/FL1 (conferred by a chlorophyll autofluorescence/green fluorescence of 2', 7'-dichlorofluorescein (DCF) for determination of oxidative stress.

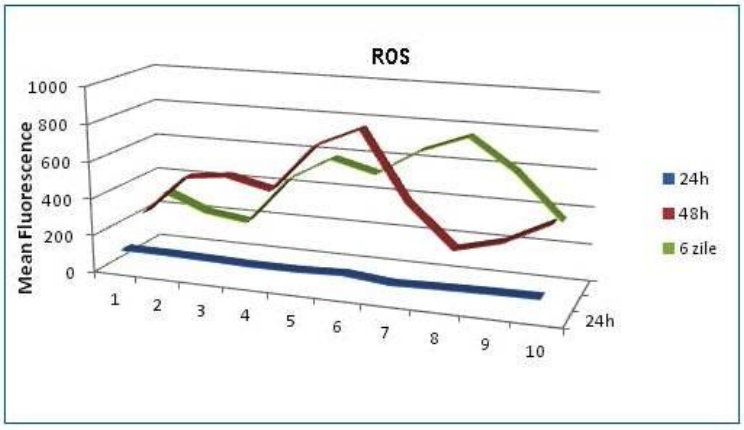

Fig. 16. Comparative analysis of the concentration of ROS induced by the action of PCP at various concentrations (1-10) after 24 hours, 48 hours and 6 days

In *Chlorella fusca*, under the actions of heavy metals ROS production is directly dependent of concentration. Particularly, high concentration generate large amount of ROS leading immediately on chlorotic cells.

Measurement of reactive oxygen species (ROS) by flow cytometry proves a sensitive method and multiparametric, which makes extremely sensitive to quantify production within the cell, with the possibility of determining its advantage on different cell subpopulations.

Determination of ROS production has the advantage to determine with great accuracy, given that free radical species have a very short lifespan.

With all these advantages, measurement of reactive oxygen species (ROS) generated under the action of heavy metals brings only information in general plan, the transformation of this idea in a bioassay, we appreciate that it is not easy.

Determination of membrane potential with DIOC6

Membrane potential and respiration rate of the *Chlorella fusca* cells were monitored using a potentiometric probe which may reveal the dynamics of changes in membrane potential between the inside and outside of the cell. Cell membrane depolarization can be detected easily lipophilic dyes such as DiBAC4 (3) or $DIOC_6$(3) (Adler *et al.*, 2007; Jamers *et al.*, 2009). The intensity of fluorescence is influenced by the penetration and removal of the fluorescent dye by the electrochemical potential of cell membrane respectively and the number of lipophilic binding sites within the cell. This type of dye, that show dependency

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load distribution can be a sensitive markers in assessing cell viability.

As probe was used $DIOC_6$ (3), dye-permeable through the cell membrane, lipophilic selectively to intracellular membrane (Gregori et al., 2002). Green fluorescence emission of this compound, with a maximum at 516 nm was collected detector FL1 (505-550 nm).

As shown in Figure 18, the analysis by flow cytometry to determine the membrane potential with DIOC6, using biparametric cytograms FL3/FL1 (autofluorescence conferred chlorophyll a/membrane potential given by the green fluorescence of $DIOC_6$), using quadrants technique identify 4 cell subpopulations, namely:

- non-chlorotic / polarized cells
- non-chlorotic / depolarized cells
- chlorotic / polarized cells
- chlorotic / depolarized cells

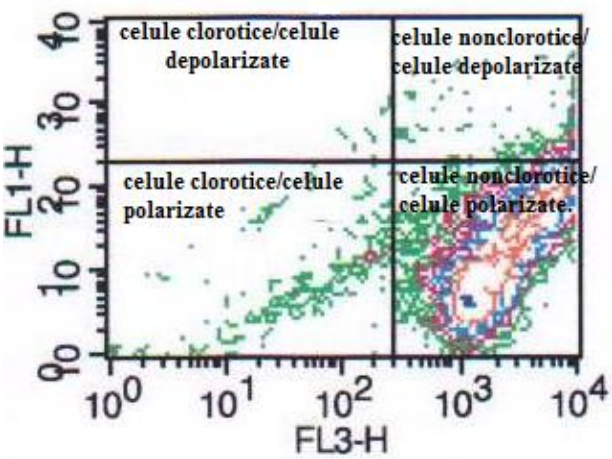

Fig. 18. Analysis by flow cytometry FL3/FL1 (conferred by the autofluorescence of chlorophyll a / green fluorescence membrane potential given by the DIOC6) for determining changes in membrane potential.

Our results showed that the heavy metals induce a depolarization of the plasma membrane in some of the analyzed microalgae population, chlorotic cells being characterized by their membrane depolarization compared to the control cells. In this way, the depolarization of the cytoplasmic membrane of *Chlorella*, after 6 days of exposure to heavy metals could be considered as an indicator of non-viable cell, such effects may be associated with alterations in the membrane caused by the peroxidation of membrane lipids as aconsequence of ROS inluence.

CONCLUSIONS:

By direct flow cytometry (cell size, cell content and chlorophyll autofluorescence) can get an analysis of cellular heterogenity (chlorotic and non-chlorotic cells) which reflects the "health" of a microalgal culture.

The analysis by flow cytometry system FSC/SSC of the *Chlorella* microalgae incubated for 6 days in the presence of various dilutions of heavy metals showed a perfect correlation of evident morphological changes observed by flow cytometry and by complementy investigation techniques, such as they were presented.

Flow cytometry has the advantage to analyze a large number of cells (10 000 cells in our case), to quantify the parameters and posibility to give a statistical presentation of the results after analysis.

In our case, the flow cytometric analysis of cytograme expressed by biparametric system FL 3/FSC (chlorophyll autofluorescence conferred/cell size) allows us to quantify the % of non-chlorotic cell in R1 and the chlorotic cells in R2. Percentages compared to control incubation lead to the possibility of tracing the "dose-response" curves for the calculation of EC50, where on the abscissa is entered the pollutant concentration and on the ordinate % of living cells, healthy, corresponding to the region R1, or respectively chlorotic cell death, corresponding to the region R2.

Determination of metabolic activity based on the esterases level of activity with Calcein-AM (cell viability assay) using a cell culture model of *Chlorella*, represents an important opportunity for determining the toxicity of pollutants due to the sensitivity and the possibility of quantifying the results.

This method correlated with other flow cytometric tests can be the basis for a flow cytometry bioassay which can be easily applied in ecotoxicity studies.

Under the action of heavy metals in *Chlorella fusca* cells occurs ROS production, depending on metal concentration, high concentrations likely generating a huge amount of ROS that lead immediately on obtaining chlorotic cells. This production is diminished with subsequent reduction of heavy metal concentration.

Measurement of reactive oxygen species (ROS) by flow cytometry proves a sensitive and multiparametric method, which makes extremely sensitive to quantify production within the cell, with the possibility of determining its advantage on different cell subpopulations.

Determination of ROS production has the advantage of a great accuracy, given that free radical species have a very short lifespan.

With all these advantages, measurement of reactive oxygen species (ROS) generated under the action of heavy metals brings general informations, but we appreciate that a bioassay based on ROS mesurement it is not easy to perform.

The results show that the heavy metals depolarization of the plasma membrane induced in some of the microalgae analyzed population, chlorotic cells are characterized by their membrane depolarization compared to the control cells. In this way, the depolarization of the *Chlorella* cytoplasmic membrane, after 6 days of exposure to heavy metals could be considered as an indicator of non-viable cell, such effects may be associated with alterations of the membrane caused by the peroxidation of membrane lipids, as a consequence of the ROS influence.

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