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FLOW CYTOMETRIC ASSESSMENT OF UNICELLULAR *CHLORELLA* CELLS ALTERATIONS UNDER HEAVY METALS EXPOSURE

Constantin-Marian PETRESCU[2], Violeta TURCUS[1,2], Daniela BRATOSIN[1,3,*]
[1]Faculty of Natural Sciences, Engineering and Informatics, "Vasile Goldis" Western University of Arad, Romania
[2]Institute of Life Sciences,"Vasile Goldis" Western University of Arad, Romania
[3]National Institute for Biological Science Research & Development (INCDSB), Bucharest, Romania

ABSTRACT. Among all organisms in aquatic ecosystems, microalgae are key targets in pollution cases, for two basic reasons: their eco-physiological similarities with terrestrial plants (the potential sensitivity of the same metabolic processes) and their role as primary producers. Any change in the proliferation of the primary producers could provoke a global alteration in the equilibrium of the aquatic ecosystems. These characteristics support the use of the freshwater microalgae in laboratory toxicological assays. Flow cytometric analyses of *Chlorella fusca* cells were performed in a FACScan analyzer or Cytomics FC 500, both equipped with an argon-ion excitation laser (488 nm), detectors of forward (FSS) and side (SSC) light scatter and fluorescence detectors. For each analyzed parameter, data were recorded in a logarithmic scale and results were expressed as mean values obtained from histograms in arbitrary units. Fluorescence of chlorophyll a (>645 nm) was used as a FCM gate to exclude non-microalgal particles. To understand the mechanisms underlying the process of cell death by heavy metals action, algal cells were exposed to different concentrations of metals (Al and Cd) and analyzed by flow cytometry for morphological changes (light scattering properties) and microscopic analysis. Our results show that microalgae are ideally suited to flow cytometric analysis and can be used and detected by flow cytometry to provide information about the physiological status of algal cells in response to toxicants.

Keywords: aquatic environment; ecotoxicological models; heavy metals; flow cytometry; ecotoxicology

INTRODUCTION

Contamination of aquatic ecosystems is characterized by long-term exposure of organisms to low doses of complex chemical mixtures. This implies the focus on developing a new methodology for assessing the ecotoxicological effects. For this reason, it is indispensable to understand molecular and cellular mechanisms through which the pollutants lead to toxic effects in organisms and finally, in populations.

Among all organisms in aquatic ecosystems, microalgae are key targets in pollution cases, for two basic reasons: their eco-physiological similarities with terrestrial plants (the potential sensitivity of the same metabolic processes) and their role as primary producers (any change in the proliferation of the primary producers could provoke a global alteration in the equilibrium of the aquatic ecosystems. These characteristics support the use of the freshwater microalgae in laboratory toxicological assays. In relation to this point, flow cytometry allows the rapid determination of a high number of cell functions by using a great variety of biochemically specific, non-toxic and fluorescent molecules in conditions close to the in vivo status in short-term exposures to high levels of light to provide information about the status of algal cells in presence of pollutants (Jochem, 2000; Molecular Probes, 2003; Gheorghe et al., 2011). Microalgae are ideally suited to flow cytometric analysis as they contain photosynthetic pigments such as chlorophyll a. In 1989, Premazzi et al., demonstrated that algal cell numbers and cell size measured by flow cytometry were similar to those obtained by counting techniques (Premazzi et al., 1989). In 1996, other groups demonstrated the usefulness of flow cytometry by investigating the effect of copper on marine diatoms (Cid et al., 1995; Cid et al., 1996). More recently, flow cytometry was applied to detect multiple effects of pollutants on algal cells at low toxicant concentrations (Franklin et al., 2001a, Franklin et al., 2001b; Franklin et al., 2002; Stauber et al., 2002). Test with marine and freshwater microalgae have been developed using flow cytometry to testing wastewaters, chemicals and sediment porewaters (Stauber et al., 2002; Hall & Cumming, 2003) and marine applications (Adams and Stauber, 2004; Adler et al., 2007). Using flow cytometric analysis, Prado et al., observed that exposure of microalga *Chlamydomonas moewusii* to increasing paraquat concentrations causing chlorosis phenomenon (Prado et al., 2011) and to develop a model for assessing the cell cycle of Chlorella vulgaris and in vivo effects of pollutants on growth and reproduction (Rioboo et al., 2009)

Consequently, to understand the mechanisms underlying the process of cell death by heavy metals action, algal cells were exposed to different concentrations of metals (Al and Cd) and analyzed by flow cytometry for morphological changes (light scattering properties) and optical and scanning microscopy.

***Correspondence:** Daniela Bratosin, National Institute for Biological Science Research & Development, Bucharest, Romania; Splaiul Independentei no. 296, district 6, Bucharest, Romania, Tel/Fax +40-(021)-2200881, email:bratosind@yahoo.com*
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MATERIALS AND METHODS

Chemicals

In the experiments we used a total of six metals: Al (NO3) 2 x 9H2O, CdO - Merck, Darmstadt, HgCl2 Sigma, Aldrich, Zn (CH3COO) 2 x 2H2O Prolabo, Pb (CH3COO) 2 x 3H2O, CuSO4 x 5H2O - Billault, Anbervilliers, France.

Microalgal cultures

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

In order to evaluate the process of alterations under heavy metals exposure, cells were exposed to different concentrations of metals, in serial dilutions, between 1000 µg/ml and 1,95 µg/ml, for 24h, 48h and 6 days at 20°C , in culture plates.

Flow cytometric analysis

Flow cytometric analysis were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest Pro software or 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. 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.

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 40C until examination. *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). Examination time was between 10 to 30 minutes, order the examination in 1500 mag, 3000 mag, 6000 mag.

RESULTS AND DICUSSIONS

Detection of altered morphology by light scattering flow cytometry

A living cell has certain characteristics that are the basis for the assessment of cell viability in flow cytometry, respectively: size or shape (FSC) and refractive (SSC), which are conditional on the level of hydration of the cell cytoskeleton and organelles condition. When a cell is altered, it begins to lose these features.

Estimation of cell viability by measuring the absorption and scattering of light, known as "direct analysis system FSC/SSC" (light scatter measurements) is very simple and sensitive. After intercepting the incident light, the cell emits a number of signals. Light scattered at an acute angle or shaft (FSC) can be correlated with cell size, thus allowing distinguishing a cell aggregates or cellular debris and assessing cell viability as dead cells diffuses less light in this direction. A right angle light scatter (SSC) allows the study of refractive cytoplasmic contents of the cell and cytoplasmic-ratio.

Changes in morphology observed by flow cytometry as "dot-plot" FSC/SSC (cell size/ granularity, density) parameters respectively quantified by XGeoMean and YGeoMean were correlated with analysis by optical and scanning electron microscopy (ESEM). Experiments were viewed at 24h, 48h, 72h and 6 days. Most obvious and representative results have been obtained at 6 days of incubation. Also, we present only the results of action and Cd on Chlorella fusca cells, which are representative of our goal.

Incubation of microalgal cells with Hg, Cu, Zn and Pb were generated similar results which bring more scientific information.

Comparative analysis by biparametric system FSC/SSC (dot plot FSC/SSC) culture of Chlorella fusca at time T0 and after 6 days incubation in the same conditions of the experiments performed without heavy metals, as shown in Figure 1, 1A and 1B demonstrate a significant change of morphology, reflected by lower values for XGeoMean, from 345.3 at 188.1 without any disruption of cellular contents. This change in cell size is confirmed by ESEM analysis, highly sensitive method, as can be seen in Exhibit 6 and the comparison samples T0a, T0B and T0c with T6 days.

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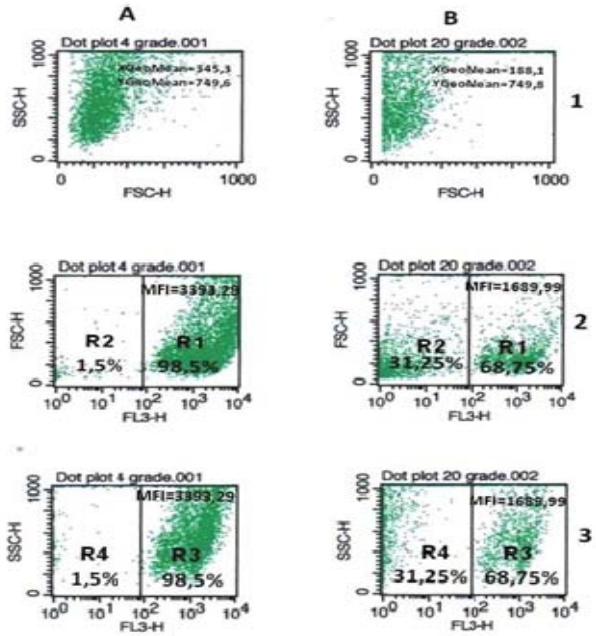

Fig.1. Comparative analysis in biparametric system (dot-plot) FSC/SSC **(1)** FL3/FSC (conferred by chlorophyll autofluorescence/cell size) **(2)** and FL3/SSC (conferred by chlorophyll autofluorescence/cell content) **(3)** microalgal *Chlorella fusca* culture at T0 **(A)** and microalgal cultured for 6 days without heavy metal **(B)**.

If we analyze the dot-plot FL3/SSC (autofluorescence conferred by chlorophyll a /content for those two witnesses (T0 and after 6 days of incubation in terms of experience without heavy metals) in Figure 1.A2 and Fig 1. B2 is found also a significant decrease in % of non-chlorotic cells in the region R1, from 98.5% to the original culture to only 68.75% after 6 days.

Comparison of dot-plots FL3/SSC (conferred by chlorophyll autofluorescence / cell content) for the 2 witnesses (T0 and T6 days after incubation in conditions of the experiments without heavy metals) in Figure 12A3 and B3 respectively, confirm the exact% of cells non-chlorotic dot-plots obtained from analysis FL3/SSC. It is noted also diminished value MFI (Mean Fluorescence Intensity) after 6 days of incubation in the conditions of the experiments performed without heavy metals, from 3393.29 to control cultures at the time T0, nearly half, ie 1689.99, which means a level reduction of chlorophyll in the cells of the non-chlorotic.

Comparative analysis by biparametric system (dot-plot) FSC/SSC for culture of *Chlorella fusca* incubated for 6 days in the presence of different concentrations of aluminum (shown in Fig. 2) clearly demonstrates changes in cellular morphology. Thus, taking into account the values of XGeoMean (cell size) and YGeoMean (cell content) of the samples of *Chlorella* incubated for 6 days at various concentrations in serial dilutions of Al compared to the control incubation without Al (T6 days) and witnessed of initial culture (T0) values refer to dot-plot analysis, we can see the following:

Compared to the blank incubation T6 days, the XGeoMean (cell size) varies only for elevated levels of Al, from 1mg/ml to 0.0625 mg / ml. This variation is manifested by decreasing's XGeoMean from 419.12 to 166.83 days for T6 incubation at the concentration of 1mg/ml. The same reduction is observed for YGeoMean (cell content) to a concentration of 1mg/ml Al up to 0.0625 mg / ml, reducing the YGeoMean from 800.31 to 306.79.

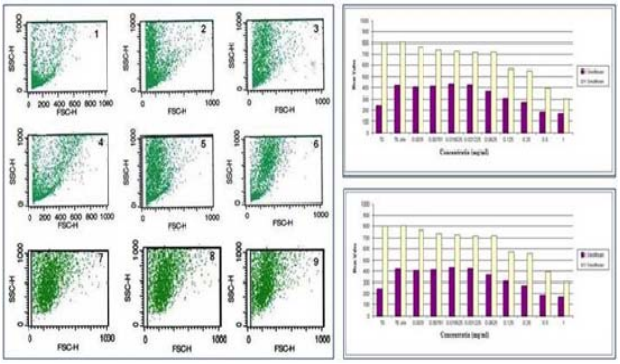

Fig. 2. Comparative analysis of biparametric system (dot-plot) FSC/SSC for *Chlorella fusca* culture incubated for 6 days in the presence of various concentrations of aluminum (1-9) and comparative histograms for XGeoMean (cell size) and YGeoMean (cell content) of the same Chlorella samples compared to the control incubation sample (T6 days) and the witness culture (T0). The results shown are representative of three experiments.

Optical and scanning electron microscopic analysis of Chlorella fusca under the action of heavy metals

All samples of *Chlorella fusca* incubated with different concentrations of Al have been analyzed by optical microscopy and scanning microscopy ESEM, representative results are shown in the following figures.

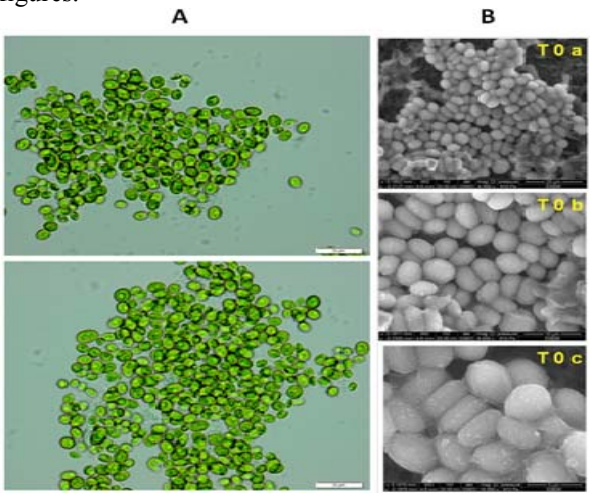

Fig.3. Analysis by optical microscopy (A) and scanning electron (ESEM) of *Chlorella fusca* culture at the time T0. The results shown are representative experiments.

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Culture of *Chlorella fusca* representing experiences at the start (T0) can be seen in Figure 3. The cells are spherical, very slightly elongated, with a relatively smooth surface, they form aggregates in the form of colonies of cells embedded in an extracellular matrix. *Chlorella* cells being blank incubation begin to undergo slight changes in morphology (Fig. 4, T24h, T48h and T6 days) or the occurrence of "swaths" or " furrows ", as shown in detail in Figure 4D. In all cases, cell colonies of incubation witnesses, T24h, T48h and T6 days, remain in the extracellular matrix.

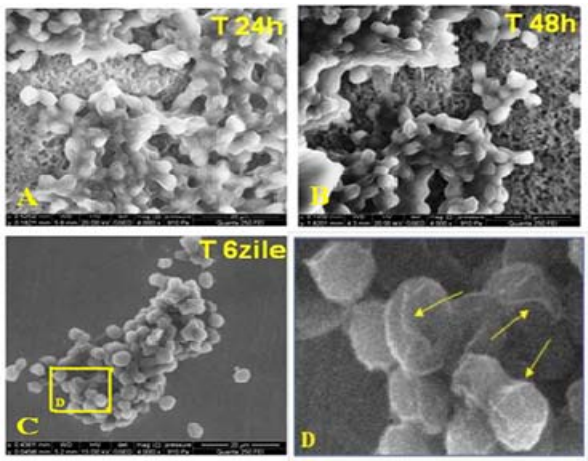

Fig.4. Scanning electron microscopy analysis of control culture of *Chlorella fusca* after 24h, 48h and 6days of incubation without heavy metals. D: detail morphology at T6 days. The arrows indicate the "furrows" on the surface of the cell wall. The results shown are representative experiments.

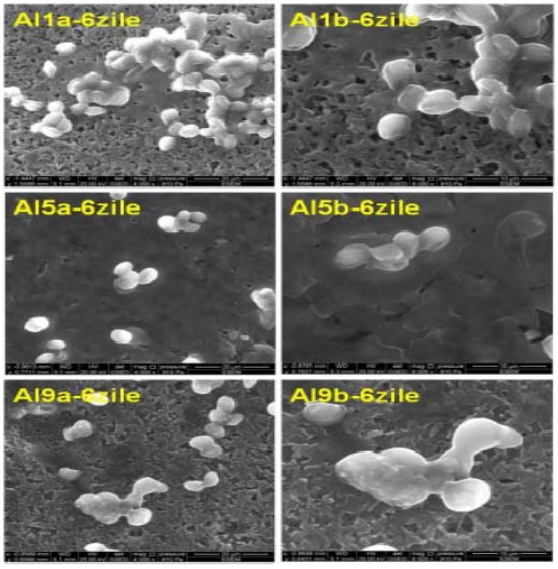

Fig. 5. Analysis by scanning electron microscopy for evidence of morphological changes of *Chlorella fusca* exposed to concentrations 1, 5 and 9 of dilution series of Al for 6 days. The results shown are representative experiments.

Under the action of aluminum for 6 days, as shown in Fig. 5 colonies diminished the waist, we observed appearance of furrows on the cell wall and more, have been observed on the surface of *Chlorella* cells "protuberances", phenotype characteristic of cells in apoptosis (Fig. 5 Al9b).

CONCLUSIONS

The direct flow cytometry (cell size, cell content and chlorophyll a autofluorescence) allows analysis of cellular heterogeneity (non-chlorotic and chlorotic cells) which reflects the default state of "health" of a culture of microalgae.

Analysis by flow cytometry in the system FSC / SSC of *Chlorella* microalgae incubated for 6 days in the presence of various dilutions of heavy metals showed a perfect correlation and highlight the morphological changes observed by flow cytometry and complementary techniques of investigation, as were presented. Incubation of cells with all heavy metals (Al, Hg, Cu, Zn and Pb) were generated similar results.

Flow cytometric analysis takes advantage of the possibility of quantification and statistical presentation of results on a large number of cells (10,000 cells in our case).

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