FLOW CYTOMETRIC AND MICROSCOPIC ANALYSIS OF GREEN ALGA *CHLORELLA FUSCA* EXPOSED TO PENTACHLOROPHENOL

C-M PETRESCU[1,2], I. STANA[1,2], C-V MIHALI[1,2], V. TURCUȘ[1,2], D. BRATOSIN[1,3]

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

***Abstract**: Pentachlorophenol (PCP) is a synthetic substance, made from other chemicals, and does not occur naturally in the environment. It is used as a pesticide The aim of this study was to track the induced changes of Chlorella fusca microalgae after exposure to various concentrations of Pentachlorophenol (PCP) by flow cytometry for morphological changes and metabolic activity and by optical and ESEM microscopy. The results reported in the present study indicate that the exposure of green alga Chlorella to Pentachlorophenol induce an apoptosis similar toxicity.*

Key words: *pentachlorophenol, algae, flow cytometry, toxicity.*

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 (PETRESCU *ET AL.*, 2013 (A); PETRESCU *et al.*, 2013 (b). Microalgae are ubiquitous and form an important component of the aquatic ecosystem.

Pentachlorophenol (PCP) is a synthetic substance, made from other chemicals, that does not occur naturally in the environment. It is used as a pesticide. It enters surface water and groundwater from factories, wood-treatment facilities, and hazardous waste sites. It also enters the soil as a result of spills, disposal at hazardous waste sites, and its use as a pesticide. Pentachlorophenol is released to the air by evaporation from treated wood surfaces and factory disposal. The disturbance of aquatic ecosystems provoked by pollution from industrial and domestic sources has as consequence the loss of biological diversity, as well as increased bioaccumulation and magnification of toxicants in the food chain.

Any interference of Pentachlorophenol (PCP) pollution with normal activities of microalgae and water could result in potentially serious consequences on the overall functioning of the ecosystem. Since algae are sensitive to pollution, any changes in their

composition may be useful as a bioindicator of pollution. Moreover, much of the experimental work on the effects of PCP on microflora have been conducted in the laboratory, while little data are available under field conditions. 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.

The present study was therefore aimed to evaluate the impact of PCP pollution towards the aquatic microflora with special reference to microalgae in polluted water and the suitability of changes in microalgal population as biological indicators of pollution.

In particular we investigated the effect of PCP on the metabolic activity based on the level of activity of esterases with Calcein-AM and microscopic analysis

MATERIAL AND METHODS

Chemicals

In the experiments we chose different concentrations of Pentachlorophenol (PCP). Pentachlorophenol (PCP- C6HCl5O) and the fluorogenic dye calcein acetoxymethyl ester (Calcein-AM), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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.

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 pentachlorophenol exposure, cells were exposed to different concentrations, 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 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. 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.

Determination of metabolic activity by flow cytometry

Determination of metabolic activity based on the level of activity of esterases has been performed with Calcein-AM. Calcein-AM is a non-fluorescent substrate permeable through cell membranes which, once entered the cell is cleaved by intracellular esterases to fluorescent product called calcein.

Calcein, the hydrolysis product of Calcein-AM substrate is a polyanionic derivative of fluorescein containing six negative and two positive charges at pH 7.0. Calcein is best retained in viable cells compared to fluorescein, carboxyfluorescein or BCECF-AM. Calcein-AM is the best fluorochrome for cell viability analysis attached to the substrate, to study chemotaxis and drug resistance. Due to its outstanding qualities, this fluorochrome it is very used in flow cytometry.

Calcein-AM is the best indicator of cell viability due to higher retention in the cell and its relative insensitivity of fluorescence emitted at pHs located in physiological limits.In viable cells with intact membranes, Calcein is retained inside, while in cells that have lost membrane integrity is removed. By flow cytometry can differentiate viable populations (which fluoresce due to the presence calcein) from nonviable (BRATOSIN *et al.*, 2005).

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

Optical and scanning electron microscopic analysis

Direct light microscopic analysis was performed using an 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). Order the examination was 1500 mag, 3000 mag, 6000 mag and examination time was between 10 to 30 minutes.

RESULTS AND DISCUSSION

Optical and scanning electron microscopic analysis of Chlorella fusca exposed to Pentachlorophenol

Changes in morphology observed in our experiments were viewed at 24h, 48h, 72h and 6 days. Most obvious and representative results have been obtained at 6 days of incubation.

Our results show a high toxicity of pentachlorophenol which translates by the existence of very small algal colonies and many chlorotic cells, or even of some solitary algae in the extracellular matrix, showing strong influence of PCP's on photosynthesis and cellular metabolism.

The most obvious are the morphological changes observed by optical microscopy as is shown in Figure 1, Figure 2, Figure 3, Figure 4 and Figure 5 at different concentrations and different times of incubation PCP.

The morphological changes observed by ESEM scanning microscopy at different PCP concentrations and different times of incubation is presented in Figure 6, Figure 7 and Figure 8

Figure 9 lead to the occurrence 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 cells, phenotype

characteristic of eukaryotic animal cells in apoptosis, and described by us under the influence of heavy metals in the scientific literature (Petrescu *et al.*, 2013).

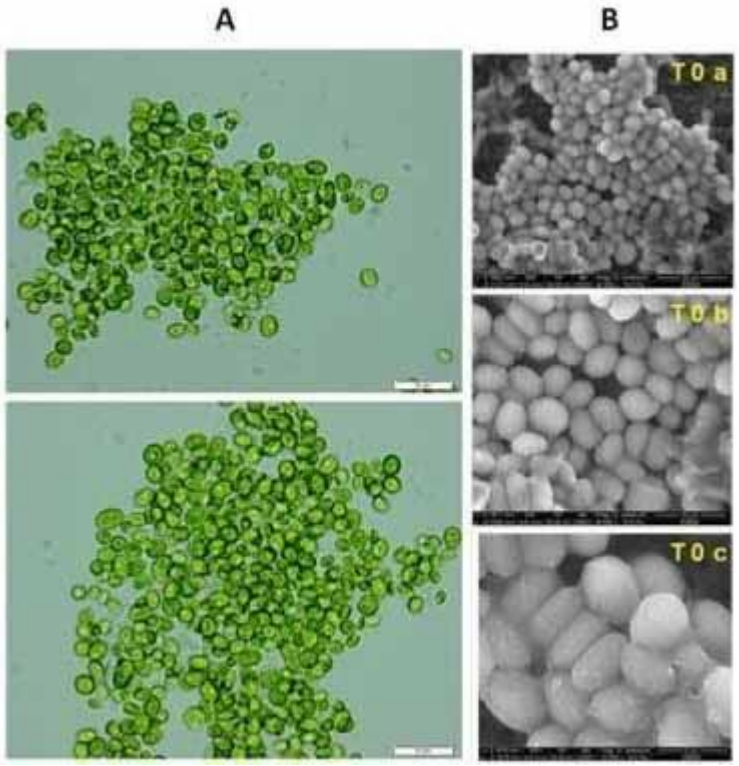


Figure 1 Analysis by optical microscopy (A) and scanning electron (ESEM) of *Chlorella fusca* culture at the time T0. The results shown are representative experiments.

A first look at the images in Figure 2, Figure 3 and Figure 4 containing optical microscopy examination of *Chlorella fusca* samples incubated with different serial dilutions of PCP at 24h, 48h and 6 days, compared with the images culture microalgae at the time T0, T24h, T48h and T6zile demonstrates a strong toxic character of Pentachlorophenol.

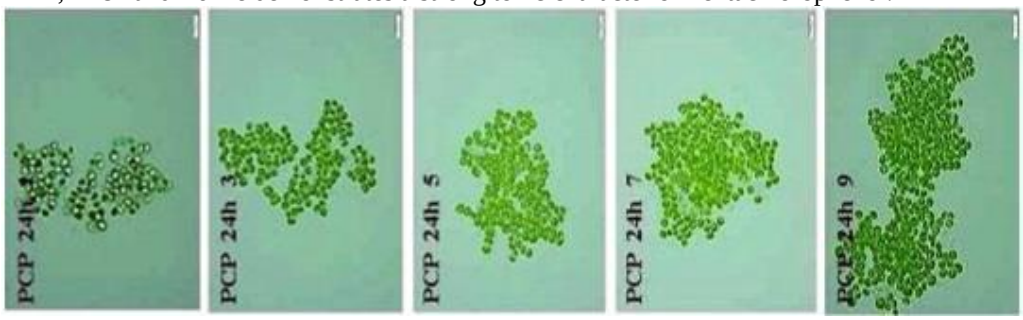


Figure 2. Optical microscopy analysis of *Chlorella fusca* culture incubation for 24 hours in the presence of various concentrations of PCP (serial dilutions 1, 3, 5, 7, 9).

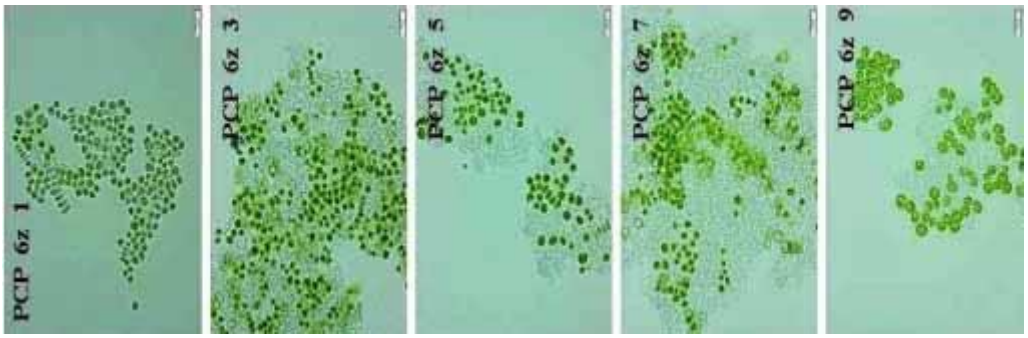


Fig. 3. Optical microscopy analysis of *Chlorella fusca* culture incubation for 48 hours in the presence of various concentrations of PCP (serial dilutions 1, 3, 5, 7, 9).

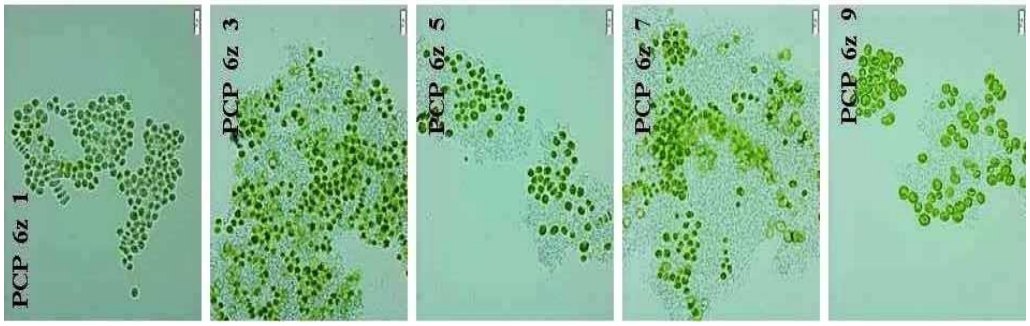


Figure 4. Optical microscopy analysis of *Chlorella fusca* culture incubation for 6 days in the presence of various concentrations of PCP (1, 3, 5, 7, 9).

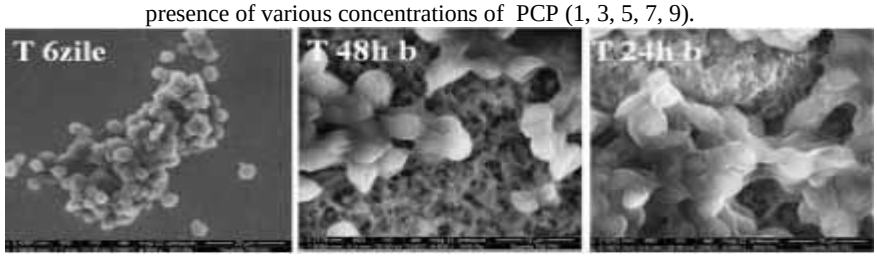


Figure 5 Analysis by scanning electron microscopy of the culture of *Chlorella fusca* control after 24 hours, 48 hours and 6 days of incubation with PCP. Indicates "furrows" on the surface of the cell wall.

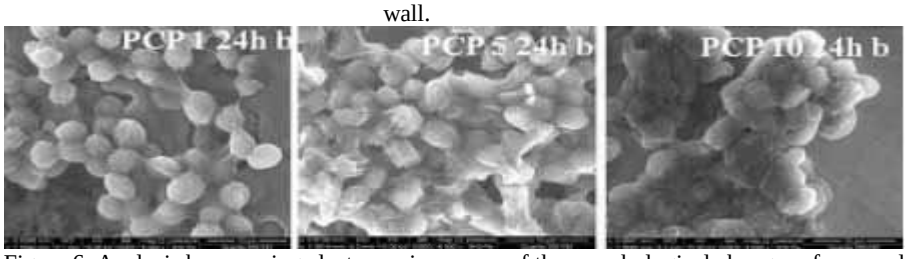


Figure 6. Analysis by scanning electron microscopy of the morphological changes of exposed *Chlorella fusca* at the concentrations of 1, 5 and 10 of the CFP (serial dilutions) for 24 hours. The results shown are representative of experiments performed.

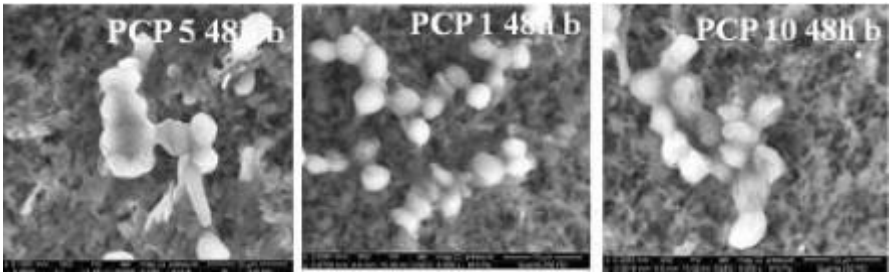


Figure 7. Analysis by scanning electron microscopy of the morphological changes of exposed *Chlorella fusca* at the concentrations of 1, 5 and 10 of the CFP (serial dilutions) for 48 hours. The results shown are representative of experiments performed.

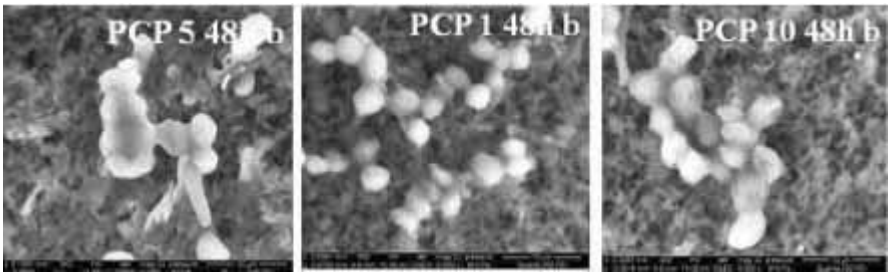
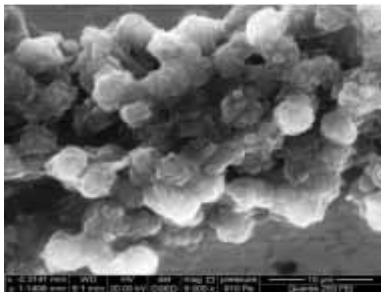


Figure 8. Analysis by scanning electron microscopy of the morphological changes of exposed *Chlorella fusca* at the concentrations of 1, 5 and 10 of the CFP (serial dilutions) for 6 days. The results shown are representative of experiments performed.



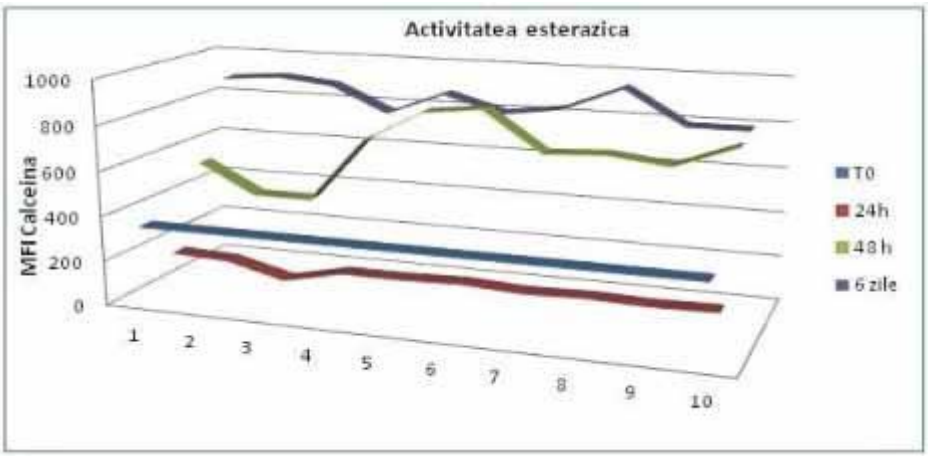


Fig. 9. Analysis by scanning electron microscope the morphology changes of *Chlorella fusca* sample the concentration of one of the CFP exposed or 0.079 g / L (0.3 mm / L PCP) for 6 days.

Determination of metabolic activity of Chlorella fusca exposed to Pentachlorophenol

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

As can be seen in figura 10, it can be noted that after 24 hours incubation of the cells with various concentrations of PCP, the values of esterases cell activity expressed as MFI (Mean Fluorescence Intensity) decrease, due to an inhibition of the enzyme, also known for other pollutants.

Unexpected is the increase in esterase activity values at 48 hours, in particular for the fifth and sixth dilutions. After 6 days of incubation, the esterase activity measured by Calcein-AM was very high, and the morphological changes were most profound.

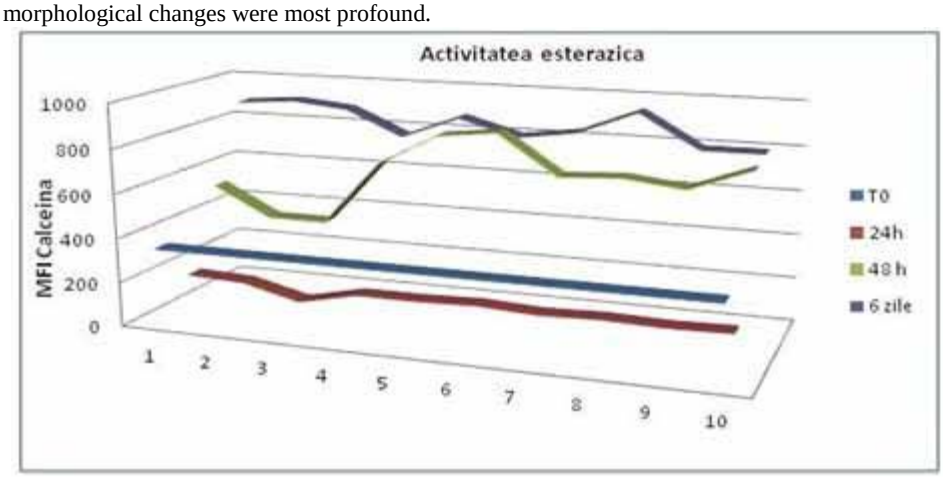


Figure 10. The comparative analysis of the values of MFI (Mean Fluorescence Intensity) obtained by determining the metabolic activity on the level of activity of intracellular esterases with Calcein-AM for samples incubated with various concentrations of PCP for 24 hours, 48 hours and 6 days.

CONCLUSIONS

From this series of experiments, we can conclude that studied Pentachlorophenol has seriously deleterious effect on *Chlorella fusca* in a dose-dependent *in vitro* and consequently this green algae is extremely vulnerable.

The results obtained by cytometric analysis have shown that the cell size, cell granularity and internal complexity were influenced by Pentachlorophenol, confirming earlier findings on ultrastructural changes on exposed microalgae.

It is emphasized the high toxicity of pentachlorophenol which is translates by the existence of very small algal colonies and many chlorotic cells, or even of some solitary algae in the extracellular matrix, showing strong influence of PCP's on photosynthesis and cellular metabolism. These changes were observed by optical microscopy and FL3/FSC system flow cytometric analysis.

Under the action of pentachlorophenol, the morphological changes observed by ESEM scanning microscopy, have been evident. At high concentrations, colonies diminished the size, appear chlorotic cells, the cell wall is observed to form "cockscombs" and more, could be seen cell of *Chlorella* with formed "protuberances" on surface. The concentration 0.079 g/L (0.3 mM PCP/L lead to the occurrence of a particular phenotype where the cells have the appearance of boiling cells, phenotype characteristic of eukaryotic animal cells in apoptosis, and described by us under the influence of heavy metals in the scientific literature for algae.(HAN *et al.*, 2006).

The metabolic activity of the algae *Chlorella fusca* with Calcein AM is deeply affected inhibited at 24h and exhibiting substantial growth at 48h, especially for dilutions 5 and 6, with an maximum of activity after 6 days of incubation, when the morphological changes were most deep.

Our results demonstrate that although very rarely used in ecotoxicology, flow cytometry is a quick and convenient technique to assess toxic effects that can generate mechanistic information on the mode of action of contaminants.

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