Use of Microalgae as Biological Indicators of Pollution: Looking for New Relevant Cytotoxicity Endpoints

Ángeles Cid, Raquel Prado, Carmen Rioboo, Paula Suarez-Bregua and Concepción Herrero

Laboratorio de Microbiología, Departamento de Biología Celular y Molecular, Facultad de Ciencias. Universidade da Coruña. Spain

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ABSTRACT

An important amount of the applied load of pesticides enter into aquatic ecosystems from agricultural runoff or leaching and, as a consequence, have become some of the organic pollutants that appear most frequently on aquatic ecosystems. The assessment of toxic potential in surface water is one of the main tasks of environmental monitoring for the contro1 of pollution. Animal organisms such as fishes or mussels have been examined intensively whereas little information is available on the susceptibility of water plants and plankton organisms.

As primary producers, microalgae constitute the first level of aquatic trophic chains. Due to its microscopic size, it is possible to get sample at population and community levels. Some species can be cultivated in photobioreactors under controlled conditions. Because of their short generation times, microalgae respond rapidly to environmental changes, and any effect on them will affect to higher trophic levels. In addition, microalgae offer the possibility to study the trans-generational effects of pollutant exposure, being a model of choice for the study of the

long term effects of pollutant exposure at population level. Furthermore, micro algal tests are generally sensitive, rapid and low-cost effective. For all these reasons, the use of microalgal toxicity tests is increasing, and today these tests are frequently required by authorities for notifications of chemicals and are also increasingly being used to manage chemical discharges. For example, algal toxicity tests of chemicals are mandatory tests for notification of chemicals in the European Union countries. Others fields of use for algae in toxicity assessment are industrial wastewaters and leachates from waste deposits.

Cytotoxic effects of aquatic pollutants on microalgae are very heterogeneous, and they are influenced by the environmental conditions and the test species. Growth, photosynthesis, chlorophyll fluorescence and others parameters reflect the toxic effects of pollutants on microalgae; however, other relevant endpoints are less known because experimental difficulties, especially under in vivo conditions.

During the last two decades, our research group has a high priority scientific objective: study the effect of different aquatic pollutants on freshwater microalgae, with the aim to develop new methods for the detection of contaminants based on the physiological response of microalgae, with the purpose of providing an early warning signal of sub lethal levels of pollution.

INTRODUCTION

Water quality issues are a major challenge that humanity is facing in the twenty-first century. Given the importance of water in public health, the growing pollution of aquatic environments as a result of rapid industrialization and intensive use of pesticides in agriculture, is a major threat to life on our planet. In this sense, there is an increasingly evident need for effective methods for assessing the toxicity of various environmental contaminants that can achieve these aquatic systems, thereby attempting to regulate the entry of potentially harmful substances to the ecosystem and, ultimately, for humans.

The problem of the impact of pollutants on ecosystems is complex. Environmental monitoring is necessary to control and reduce this impact, and all aspects (legal, social, economic and biological) should be considered. Given the need for appropriate and effective methods for assessing the toxicity of different pollutants, microorganisms, and in particular microalgae, have begun to be used as biological indicators of pollution in ecotoxicity studies because of its predominant role in the first level of the food chain. The aim of these studies is to control entry into the ecosystem of substances potentially harmful to life in them.

The most common ecotoxicity assays for monitoring aquatic pollutants are short-term lethality tests on fishes, which have been criticized for economic, logistical and ethical reasons (Fentem and Balls, 1993). One alternative proposed is the use of lower level organisms of the aquatic food chain, such as bacteria and micro algae, which would be beneficial from all points of view raised. Microalgal bioassays conducted in the laboratory may help to give valuable information on the effects of these pollutants, so the management or environmental advice in these organisms have an early and appropriate alarm system that would allow decision-making prevent such effects.

The growing awareness of the harmful effects of pollution on the environment has changed the traditional strategy of seeking ways to restore or environmental recovery towards a strategy for preventing the entry of potential environmental contamination. This change of attitude must lead scientific community to investigate new methods to predict the toxicity of various substances that can be released into the environment and can exercise potential damage to the ecosystem. There is also a growing demand for test methods increasingly sensitive.

The history of bio logical assays began in 1924 when Carpenter (1924) began studying metal toxicity to fish in a mining area. Bioassays with algae began to use more than three decades ago, and the first job held in 1971 (Burrows, 1971). These toxicity tests with algae have been becoming more important, so that algal groVl1h inhibition tests are now included in the toxicity bioassays required for registration and notification of new chemicals in the European Union (Girling et al., 2000; Pascoe et al., 2000).

MICROALGAL (UNIALGAL) BIOASSAYS

Microalgae are a diverse group - of phototrophic microorganisms found in most environments, especially in the water bodies, both freshwater and saltwater. In these aquatic environments are the main primary producers and represent, therefore, the main energy input to the ecosystem. Thus, any disturbance in the microalgal population and! or alteration of primary production can impact severely on other organisms in these environments (Campanella et al., 2001; Lülrling and Roessink, 2006).

Nowadays, microalgae are considered useful indicators of environmental quality, thanks to some of its characteristics: they are ubiquitous inhabitants of all water bodies; it is undeniable they are representative members of the phytoplankton; in general, they are easily cultivated in the laboratory; and they are sensitive to a broad group of compounds, both organic and inorganic. Therefore, microalgae are commonly used in laboratory bioassays (McCormick and Cairns, 1994; Nie et al., 2009).

However, the algae are still underrepresented as test organisms in standardized methods recommended, and since there is no species of microalgae that are always the most sensitive and ecologically representative, it is necessary to introduce new microalgal species that can be used toxicity bioassays, so that in each case to choose the most appropriate, taking into account the nature of the aquatic environment to be protected and the organisms that live naturally in the middle so that the ecological significance of tests in the laboratory is growing.

Laboratory bioassays using a single microalgal species are the most common, and usually required, to evaluate the toxicity of new substances that seek entry into the market. Thus, laboratory bioassays with microalgae is increasingly used to evaluate the toxicity of chemical compounds and pollutants are part of the strategies recommended by the European Community Commission and the Environmental Protection Agency for U.S. damage assessment of toxic agents (Petersen and Kusk, 2000).

Unialgal bioassays conducted in the laboratory can be performed using batch cultures, in which the toxicant is added to the assay and there is no renewal of the culture medium or regulation of the concentration of pollutant, or continuous cultures, in which there is a renewal of culture medium at a certain rate.

On the other hand, are increasingly being used more batteries of bioassays performed at the same time and independently with different species of micro algae but with the same toxic. These batteries can thus compare the sensitivity of different micro algal species, or even different strains of the same species, to a particular pollutant.

Since different species of microalgae differ markedly in their responses to toxic agents, the bioassays using a single microalgal species are of limited applicability in assessing the effects of these environmental pollutants on algal communities, which consist of several species different sensitivities. However, these toxicity tests have been the source of many biological data for risk assessment of different pollutants and there are authors who believe, like us, that these bioassays can still provide valuable information on this subject (Ma, 2005).

There are relatively few microalgal species have been studied for use in toxicity tests, and those used have been chosen for its ease of cultivation rather than by their sensitivity to pollutants. Therefore, there is a need to seek new and more sensitive species respond to both promoters and growth inhibitors.

Most toxicity tests with microalgae performed in laboratory have been conducted with freshwater microalgae, and there are relatively few bioassays that can be classified as standard in marine environments. It is well known that green algae and cyanobacteria are relatively sensitive to many chemicals. The green microalgae of the genera *Chlamydomonas, Chlorella,*

Scenedesmus and *Selenastrum* are frequently used in bioassays of the toxicity of different contaminants. In particular, the most widely used microalgal bioassay is based on inhibiting the growth of algae *Selenastrum capricornutum* and *Scenedesmus subspicatus* (ISO 8692, 1989). There are also many studies conducted with various species of cyanobacteria, being *Anabaena*, *Nostoc* and *Microcystis* the most common genera in these studies. On the other hand, their ecological position (primary producers) and their essential roles in recycling nutrients are critical for all environments. In addition, knowledge of the processes of absorption, accumulation and metabolism of contaminants by algae is essential, as they play an essential role in the process of biomagnification of these contaminants along the food chain, which may ultimately lead to mortality fish, birds and mammals.

MICROALGAL PARAMETERS APPLIED ON TOXICITY STUDIES

Growth

Growth is the most studied parameter in toxicity tests with microalgae, so that 95% or more of the published works include it. It is a very general parameter, reflecting the physiological state of cells. Microalgal population growth can be monitored directly by counting cells under a microscope in special chambers, or electronic particle counters, or using flow cytometry. Indirect estimates of growth can also be used that can be correlated to the turbidity of the culture, the dry weight or the amount of chlorophyll *a* (by fluorometry or spectrophotometry).

There are different index or rates, usually based on the results of cell density, allowing us to quantify the effect of pesticide on microalgal growth, the most used growth rate (μ) and the median effective concentration (Median Effective Concentration, Effective Concentration 50%, EC_{50}) which is the concentration of toxic compound that reduces population growth by 50%. The easiest way to obtain an EC value is the graphic interpolation.

In contrast, very little is known about growth and proliferation in relation to the cell cycle regulation of algae. The lack of knowledge is even greater when referring to the potential toxic effects of pollutants on microalgal cell division. To assess the effect of terbutryn, a triazine herbicide, on the proliferation of the freshwater microalga *Chlorella vulgaris* we have used a flow cytometric approach; *in vivo* cell division was followed using 5-,6- carboxyfiuorescein diacetate succinimidyl ester (CFSE) as staining (Rioboo et al., 2009a). In all C. *vulgaris* cultures, each mother cell had undergone only one round of division through the 96 h of assay and the cell division occurred during the dark period. Cell division of the cultures exposed to the herbicide was asynchronous, and terbutryn altered the normal number of daughter cells (4 autospores) obtained from each mother cell. The number was only two in the cultures treated

with 250 nM. The duration of the lag phase after the exposure to terbutryn could be dependent on the existence of a critical cell size to activate cytoplasmic division.

The rapid and precise determination of cell proliferation by CFSE staining has allowed develop a model for assessing both the cell cycle of C. *vulgaris* and the *in vivo* effects of pollutants on growth and reproduction at micro algal cell level (Rioboo et al. 2009a).

Cell Viability

An effective method to determine cell viability is to measure the fluorescence of cells stained with propidium iodide by microscopy or flow cytometry. Propidium iodide is a fluorescent dye that penetrates cells when they die and/or the cell membrane integrity is lost, this fluorochrome fluoresces red when excited with blue light. Thus, it can be used to discriminate between viable cells and cells not viable fluorescent fluorescent (Cid et al., 1996; Franqueira et al., 2000).

Some studies have also used staining with fluorescein diacetate as a test to assess cell viability, based on the fluorescein molecule, resulting from the conversion of the compound by nonspecific intracellular esterases, is polar in nature and is retained in cells that have intact membrane; however, cells that have lost membrane integrity showed no green fluorescence characteristic of the fluorophore (Lage et al., 2001).

Taking into account our results, cell viability assayed by flow cytometry was the less sensitive parameter when the marine diatom *Phaeodactylum tricornutum* was exposed to copper concentrations lower than 1 mg l^{-1}, during 24 hours (Cid et al. 1997). However, cell viability is a good indicator for the selection of microalgal species with bioremediation purposes (Gonzalez-Barreiro et al., 2006).

Elemental and Biochemical Composition of the Microalgal Biomass

The metabolites can be considered as the end products of cellular regulatory processes and their levels can be interpreted as the ultimate response of biological systems to genetic and environmental changes (Jamers et al., 2009). A very basic index used is the C/N ratio, which has been linked with a growth rate inversely proportional (Laws and Chalup, 1991).

The biochemical composition analysis to characterize the metabolic response of an organism to stimuli or stressors in the environment has been little used so far in microalgae. Different biochemical compounds have been used as study parameters in toxicity tests with microalgae, such as the cellular protein content (Battah et al., 2001), the carbohydrate content of cells (Kobbia et al., 2001), cellular lipid content (Yang et al., 2002) or fatty acids (ElSheekh et al., 1994). The cellular content of various photosynthetic pigments, mainly chlorophylls and carotenoids (Couderchet and Vernet, 2003), are also related to the photosynthetic activity.

In our investigations we have observed that atrazine exposure induced the process of chlorosis in cyanobacterial cells, given that this herbicide has an effect on photosynthesis, chlorotic subpopulations having low values of chlorophyll *a* autofluorescence (Gonzalez Barreiro et al., 2004). More unpigmented subpopulations (chlorotic) appeared as the atrazine concentration increased and better growth rates resulted.

We have also detected that the herbicide paraquat induces alterations in the elemental and biochemical composition of a non-target microalgal species, *Chlamydomonas moewusii* (Prado et al., 2009b). After 48 h of herbicide exposure, growth rate, dry weight, and chlorophyll *a* and protein content were affected by paraquat concentrations above 0.05 ~M. C/N ratio was also affected due to a decrease in nitrogen content in the dry biomass, while the carbon content remained constant for all paraquat concentrations assayed.

The analysis of the photosynthetic pigment content of C. *moewusii*, using a traditional spectrophotometric technique that provides population bulk measurements, indicated us an alteration provoked by paraquat (Prado et al., 2011). By means of flow cytometry, which allowed us characterizing the microalgal response at a single-cell level, we have observed that paraquat concentrations above 50nM induce chlorosis in a percentage of microalgal cells depending on herbicide concentration and exposure time, as reflected by a reduced cell chlorophyll autofluorescence and pigment content of the biomass. The possibility of analyzing chlorotic and non-chlorotic sub-populations separately allowed us the study of morphological properties and physiological status of both cell types, leading to the conclusion that chlorotic cells are non-viable cells. Chlorophyll fluorescence was the most sensitive parameter since even cells exposed to the lowest concentration assayed, 50nM, although not chlorotic, showed a significantly reduced chlorophyll fluorescence with respect to control cells, reflected also by a reduced chlorophyll content of the biomass (Prado et al., 20 11).

We have also analyzed the pigment profile of *Phaeodactylum tricornutum* cells exposed to copper (Cid et al., 1995). In this study, the increase of the intracellular pH provoked by the presence of copper induced the alteration of the proportion of the chlorophyll allomers.

Changes in Cell Morphology

Exposure of microalgal cells to pollutants, such as metals or pesticides, can induce changes in cell morphology, both in terms of volume and shape of the cells as produced at the subcellular level changes (changes in the morphology of chloroplasts and mitochondria, appearance of cytoplasmic inclusions, alteration of membrane, and others). These structural and ultrastructural changes can be studied by light and electron microscopy (Torres et al. 2000; Yang et al., 2002) or using flow cytometry (Abalde et al., 1995; Cid et al., 1995; Rioboo et al., 2002).

Cadmium caused ultrastructural changes in *Phaeodactylum tricornutum* cells: deposition of metal on the cell surface, increase of the chloroplast volume, appearance of electrodense granulations, and reduction of lipid inclusions (Torres et al., 2000).

Chlamydomonas moewusii cells exposed to the herbicide paraquat concentrations higher than 0, 15 µM, formed palmelloid colonies (clusters of non-flagellated cells closed in a common wall), observed by light microscopy, and the cellular volume and complexity analysed by flow cytometry technique increases, and this fact could be related to palmelloid colony formation, probably due to the incapacity to finish cell division as well as failures of regulation of cell volume because of the attack of oxidative radicals formed to membranes (Franqueira et al., 1999; Rioboo et al., 2008).

Cell Physiology

The toxicity exerted by environmental pollutants may be reflected in different enzyme activities, which can be induced or inhibited by the presence of the toxic agent. Enzyme inhibition measurements in microalgae are becoming increasingly popular indicators of environmental stress because they offer a rapid and sensitive end point. Generally tend to study enzymatic activities related to antioxidative mechanisms (catalase, peroxidase, glutathione reductase), as environmental contaminants typically trigger oxidative stress mechanisms may induce antioxidative enzymatic and non-enzymatic, which thus can be used as biomarkers of toxicity (Geoffroy et al., 2002).

Other enzymatic activities were also studied, such as non-specific esterase activity, usually using the fluorogenic substrate fluoresce in diacetate, which is transformed into the fluorescent compound fluorescein by esterases, so that measuring the fluorescence resulting from the reaction can be determined the activity of these enzymes (Prado et al. 2009a). On the other hand, there are also studies of particular enzymatic activities, including various enzymes related to nitrogen metabolism. Enzymes involved in nitrogen assimilation were affected by the herbicide paraquat, being nitrate reductase activity more sensitive to paraquat than nitrite reductase, leading a significant alteration of the C/N ratio (Prado et al., 2009b).

In the case of the microalgae *Dunaliella tertiolecta,* whose growth is not a particularly sensitive parameter (Abalde et al., 1995) found that a bioassay of toxicity of various pollutants based on the activity of β-galactosidase enzyme is more sensitive than traditional tests of growth inhibition, this enzyme bioassay is rapid, sensitive and reproducible, and correlates well with other ecologically relevant parameters such as growth (Peterson and Stauber, 1996).

The photosynthetic activity can be assessed by different methods, two of the more traditional methods of assimilation of ^{14}C-radiolabeled CO_2 and monitoring of the evolution of O_2 through

electrodes as the type Clark. Since photosynthesis is one of the most common targets of pollutants, especially herbicides, the use of this physiological parameter in toxicity tests with microalgae is quite common. Inhibition of photosynthesis rapidly reflects the toxic effect of different pollutants on microalgae (Cid et al., 1995; Macinnis-Ng and Ralph, 2003; Strom et al., 2009).

A relatively short time has begun using chlorophyll fluorescence as an effective indicator of the physiological state of the photosynthetic apparatus, providing basic information on the functioning of photosynthesis. When a photosynthetic organism is exposed to light, it produces a fluorescence emission originating mainly from chlorophyll a of photosystem II.

Photosynthesis and fluorescence are competing processes, so if microalgal cells adapted to darkness are illuminated, the fluorescence rapidly reaches a maximum and begins to decline with the onset of electron transport (Kautsky effect) (Mallick and Mohn, 2003). Under optimal conditions, most of the light energy absorbed by chlorophyll is dissipated via chemical conversion with a small proportion devoted to the emission of fluorescence and heat, but the photosynthetic capacity of the organism may be reduced under stress conditions, giving lead to an increase in fluorescence emission. This has been observed in the case of microalgae exposed to herbicides inhibiting electron transport at the photosystem II level (Ellaffroy and Vernet, 2003). Compared with the traditional method of assimilation of $^{14}CO_2$, the measurement of chlorophyll fluorescence to evaluate the toxicity of pollutants offers several advantages: it is done quickly, so non-invasive, without incubation, without bottle effects without radioactivity and without destroying the integrity of the cells. Additionally, you can determine various parameters related to fluorescence, which can give an idea of the primary mode of action of pollutant.

The effect of copper on photosynthesis and related parameters of a marine diatom was analyzed in our laboratory; a copper concentration of 0,5 mg l^{-1} reduced in a 50% the photosynthetic rate of *Phaeodactylum tricornutum,* measured as radioactive carbon assimilation, whereas a concentration of 0.10 mg l^{-1} is needed to reduce in a 50% the growth rate, and 0,05 mg l^{-1} of copper provoked a significant decrease in the cellular pool of ATP (Cid et al. 1995). The effect of copper on the pigment profile was mentioned above.

Damage at the DNA Level

It has been observed that many of the toxic agents that contaminate aquatic environments are able to interact with the DNA of living cells, causing genotoxic effects, so that the study of the genotoxic potential of these pollutants has become one of the main objectives of bioassays for pollution control in aquatic systems.

Genotoxic effects of environmental contaminants can be tested using a wide range of tests based on biomarkers (Ali and Kumar, 2008), but recently, and for toxicity tests with microalgae, has gained increasing importance the "comet assay", since it has been revealed as a simple, rapid and sensitive for determining genotoxicity and evaluating level of damage to the structure of DNA (Akcha et al., 2008; Li et al., 2009). This test enables the detection of various DNA lesions, such as the presence of breaks in one strand, induced by physical or chemical agents, allowing the study of each cell separately and enabling the establishment of intercellular differences in the population. In addition, this method is applicable to any eukaryotic cell and is independent of cell proliferation (Erbes et al., 1997).

The comet assay is based on single-stranded DNA breaks induced directly by genotoxic agent or as a result of alkali treatment causes the formation of so-called "comets" after DNA migration in electrophoresis in basic media. After staining the DNA with a fluorophore such as ethidium bromide or SYBR Green, damaged cells appear as comets consist of a head and a tail heavily stained diffuse DNA fragments that have migrated because of its small size. This test can detect DNA damage induced by alkylating agents, intercalating or as a consequence of oxidative stress (Henderson et al., 1998).

Potential DNA damage assayed by the comet assay, provoked by the herbicide paraquat on the freshwater microalga *Chlamydomonas moevvusii* was also studied in our laboratory (Prado et al., 2009a). After only 24 h of herbicide exposure significant DNA damage was observed in microalgal cells exposed to all paraquat concentrations assayed, with a 23.67% of comets in cultures exposed to 0.05 µM, revealing the genotoxicity of this herbicide (Prado et al., 2009a).

Other Cytotoxicity Endpoints

Flow cytometry has been applied in the study of the aquatic environment since the 19 80s; this technique has achieved extensive use in the study of microalgae and this technique has been introduced as an alternative to the more traditional techniques of analysing cells in culture and from natural populations (Franqueira et al., 2000).

Aerobic organisms produce reactive oxygen species (ROS) in their metabolic process, as oxygen peroxide 0 superoxide. The level of these ROS increases during several cytotoxic process, leading to the named oxidative stress. The fluorogenic oxidation of hydroethidine (dihydroethidium; HE) to ethidium has been used as a measure of O2-. Microalgal suspensions were stained with hydroethidine (HE). HE is a chemically reduced fluorophore able to cross the cell membrane and it is oxidized by superoxide ion in the cytoplasm of cells to a red product (ethidium) (Benov et al., 1998). Ethidium binds to the DNA inside cells and has a red fluorescence when is excited with blue light (Shapiro, 1995). As we have demonstrate,

Chlorella vulgaris cells exposed to 500 nM ofterbutryn, showed an important increase of the oxidative stress level, respect to control cells, after 96 h of exposure (Rioboo et al., 2009b).

Membranes could be expected to be highly prone to free radical attack inasmuch as unsaturated fatty acids are major components of most membrane lipid bilayers (Cid et al., 1996). The consequences of free radical attack on membranes are numerous and include the induction of lipid peroxidation (Kellog and Fridovich, 1975), lysis (Goldstein and Weissmann, 1977), and fatty acid de esterification (Niehaus, 1978). Senescence is an active process initiated by some combination of internal and environmental triggers, and membrane deterioration is an early and fundamental feature of this process.

Calcium ion plays an important role as a mediator in the transmembrane signal transduction, being its intracellular concentration increase part of the regulation of several cell processes (Tsien, et al, 1982). The development of fluorescent probes that show a spectral response upon binding Ca^{2+} have enabled researchers to investigate changes in intracellular free Ca^{2+} concentrations (Tepikin, 2001). These fluorescent indicators are derivatives of Ca^{2+} chelators. Fluo-3 is a Ca^{2+} fluorescent indicator excited with visible light and it is essentially non-fluorescent unless bound to Ca^{2+} After its union to Ca^{2+}, Fluo-3 increases its fluorescence between 100 and 200 times (Burchiel et al., 2000). Using the flow cytometry technique, we have seen that the intracellular calcium level is closely related with the photosynthetic metabolism of control cultures cells, since the $[Ca^{2+}]_i$ increases in darkness and decrease in the light period as be found in *Chlorella vulgaris* (Rioboo et al., 2009b).

However, those cells treated with 500 nM of terbutryn showed a drastically different pattern, remaining more or less constant the level of calcium after the first light period (Rioboo et al., 2009b). This terbutryn effect confirms that changes in the intracellular calcium level in this kind of cells are a consequence of the alteration in the photosynthetic process, and an increase in the $[Ca^{2+}]_i$ can be interpreted as an early signal of cell stress.

Microorganisms in general and microalgae in particular, are the first organisms affected by pollutants discharges in aquatic environments because they are directly in contact with the medium, separated only by the cytoplasmic membrane and the cell wall. Cellular membranes are selective, dynamic barriers that play an essential role in regulating biochemical and physiological events, so any alteration produced in the environment provokes changes in microorganism membranes. Potentiometric optical probes enable researchers to perform membrane potential measurements in cells (or organelles) too small for microelectrodes. The plasma membrane of a cell typically has a transmembrane potential as a consequence of K^+, Na^+ and CL^- concentration gradients that are maintained by active transport processes.

Potentiometric probes offer an indirect method of detecting the translocation of these ions. Increases and decreases in membrane potential -referred to as membrane hyperpolarization and depolarization, respectively- play a central role in many physiological processes, including cell signalling.

$DiBAC_4(3)$ (bis-(l,3-dibutylbarbituric acid trimethine oxonol) is a slow-response probe, which exhibits potential-dependent changes in its transmembrane distribution that is accompanied by a fluorescence change. The dye enters depolarized cells where it binds to intracellular lipids. Increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. This dye is excluded from mitochondria because its overall negative charge, simplifying the membrane potential measurement in eukaryotic organisms (Jepras et al. 1995). We have observed a decrease of $DiBAC_4(3)$ fluorescence respect to control cells can be observed in *Chlorella vulgaris* cells exposed to 500 nM terbutryn after 96 hours (Rioboo et al., 2009b), indicating the hyperpolarization of the cell membrane of affected cells.

CONCLUSION

It has been confirmed that inhibition of growth and photosynthesis, as well as other variables closely related to photosynthesis (A TP formation, radioactive carbon assimilation, oxygen evolution and algal fluorescence induction phenomena) reflect the toxic effects of pollutants on microalgae. Nevertheless, other relevant endpoints are less known because experimental difficulties, especially under *in vivo* conditions. The variety of the results obtained in our laboratory using the flow cytometry technique allowed us to indicate new methods for the detection of several pollutants toxicity, based on the physiological response of micro algae, with the purpose of providing an early warning signal of sublethal levels of pollution.

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