Use of the Comet Assay to Evaluate Pesticide Toxicity on Non-Target Microalgae

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ABSTRACT

Aquatic environments are often contaminated with numerous organic and inorganic compounds. More than 90% of the applied load of pesticides is dispersed in the environment, reaching non-target species of these aquatic systems. Several of these compounds are reported to be capable of interacting with the DNA of living cells and therefore cause genotoxic effects. The assessment of genotoxic potential in surface water is one of the main tasks of environmental monitoring for the control of pollution. Alkaline single-cell gel electrophoresis, also known as the comet assay, is a valuable technique which allows detection of DNA damage at single-cell level and provides a unique opportunity to investigate intercellular differences.

The potential genotoxicity of herbicides on non-target freshwater microalgae using the comet assay was studied. Taking into account the results obtained, comet assay provides a sensitive and rapid system for measuring primary DNA damage in microalgae which could be an important aspect of environmental genotoxicity monitoring in surface waters.

Aquatic environments are often contaminated with numerous organic and inorganic compounds. More than 90% of the applied load of pesticides is dispersed in the environment, reaching non-target species of these aquatic systems. Several of these compounds are reported to be capable of interacting with the DNA of living cells and therefore cause genotoxic effects. The assessment of genotoxic potential in surface water is one of the main tasks of environmental monitoring for the control 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, phytoplanktonic 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. Due to a high growth rate, microalgae offer the possibility to study the transgenerational effects of pollutant exposure, being a model of choice for the study of the long term effects of pollutant exposure at population level (McCormick and Cairns, 1994; Akcha et al., 2008).

Most phytotoxicological research with herbicides has been conducted on target plants. The sensitivity of algae to many herbicides is very high, and a better understanding of their environmental effects is probably acquired by using test species representing non-target groups (Haglund, 1997). Because of their short generation times, rnicroalgae respond rapidly to environmental changes, and any effect on them will affect to higher trophic levels (Ma et al., 2002, Rioboo et al., 2007). Furthermore, microalgal tests are generally sensitive, rapid and low-cost effective (Sosak-Swiderska et al., 1998). For 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 (Mayer et al., 1997; *Rioboo* et al.,2002).

Up to now, most of the genotoxicity studies conducted in microalgae focussed on the formation of cyclobutane pyrimidine dimers, a DNA damage typical from UV irradiation (Fafandel et al., 2001; Häder and Sinha, 2005).

Alkaline single-cell gel electrophoresis, also known as the comet assay, is reported as a useful and sensitive technique for genotoxicity monitoring. It is a valuable technique which allows detection of DNA damage at single cell level and pro vides a unique opportunity to investigate intercellular differences (Desai et al., 2006). This assay enables sensitive detection of DNA lesions including single-strand breaks, alkali-labile sites and other modifications of the molecules, induced in cells by physical and chemical agents. Under alkaline conditions, DNA base-pairing is disrupted. Single-strand breaks directly induced by DNA-damaging substances or subsequent to alkaline treatment cause the formation of so-called "comets" after DNA migration in electrophoresis. After DNA staining with a fluorescent dye, damaged cells appear

as comets composed of an intensively stained comet head and a tail of migrated DNA fragments. The comet assay is able to detect DNA damage induced by alkylating agents, intercalating agents, and oxidative damage (Henderson et al., 1998). Because of the simple procedures, high sensitivity, short response time and the requirement of relatively small number of cel1s and test substances, it has been a powerful tool for the determination of genotoxicity (Aoyama et al., 2003; Avishai et al., 2003). Furthermore, trustest method is applicable to any kind of eukaryotic cell and is independent of proliferation or cel1 cycle status (Erbes et al., 1997).

Erbes et al. (1997) demonstrated that a test protocol of the single-cell gel electrophoresis often used (Singh et al., 1988) could be applied to detect primary DNA damage in the planktonic alga *Chlamydomonas reinhardtii,* after some modifications. These modifications consist of using an alkaline lysis buffer with ionic detergents, in order to lyse sufficiently the cells of *Chlamydomonas* in spite of its strong cell wall formed by three glycoprotein layers (Roberts et al., 1972; Goodenough and Heuser, 1985; Van den Hoek et al., 1995), and reducing preincubation and electrophoresis times in order to make visible the low genomic DNA content per vegetative haploid cell (Harris, 1989).

The potential genotoxicity of the herbicide paraquat was assessed in our laboratory on, the freshwater green microalga *Chlamydomonas moewusii* (Prado et al., 2009), using the comet assay counting the number of comet cells against total normal cells (Figure 1). *Chlamydomonas* was previously reported as a suitable microalgal species for this assay (Erbes et al., 1997). The use of a microalgal test species for the comet assay must be validated after an analysis following H_2O_2 exposure, where a significant dose-dependent increase in comet parameters must be observed. Some microalgal species present nuclei too small in size to be correctly scored by normal image analysis systems (Desai et al., 2006; Akcha et al., 2008).
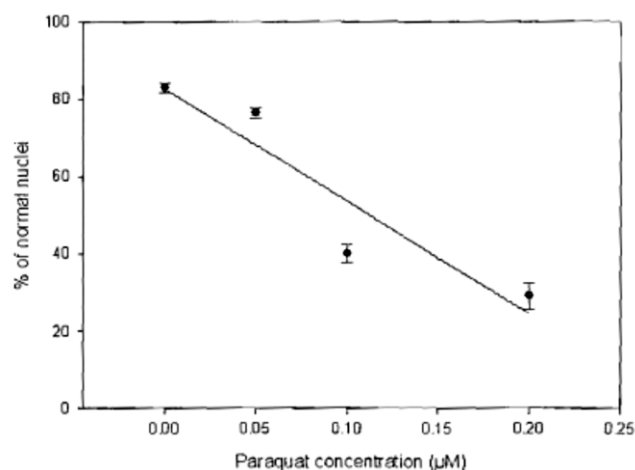

Figure l. Relationship between the percentage of normal nuclei cells and the paraquat concentration in the medium, expressed as μM (r = 0,93).

The aim of the mentioned study of Prado et al. (2009) was to compare data obtained using different techniques on the basis of affected *vs.* non affected cells; then, there was not necessary any other interpretation of results than this. Although the comet assay methodology is straightforward and does not require sophisticated equipment, the analysis of the comet, images is not so simple. We have used a visual analysis, classifying nuclei on the basis of their morphology in two groups: comets and normal nuclei (Figure 2). DNA damage is evaluated as an increase in the percentage of cells with comets (Devaux et al., 1997; Avishai ,et al., 2002; Desai et al., 2006). This comet scoring (% of comets, or normal nuclei) could be improved with the technical possibilities and the conclusions of the International Workshop on Genotoxicity Test Procedures at San Francisco in 2005 where image analysis is recommended, with the measurements of parameters such as the percentage of DNA in the tail (percent migrated DNA), tail length and tail moment (fraction of migrated DNA multiplied by some measure of tail length). More information on this more exhaustive scoring of the comet assay results, can be found in Hartmann et al. (2003) and http://www.scorecomets.com/assay/hints.php .

Results obtained in our assay showed that paraquat induces DNA damage in cells of *Chlamydomonas moewusii,* after only 24 hours exposed to paraquat concentrations of 0.05 μM or higher (Prado et al., 2009), in a dose-dependent manner, decreasing the percentage of normal nuclei cells as the paraquat concentration increases in the medium (Figure 1).
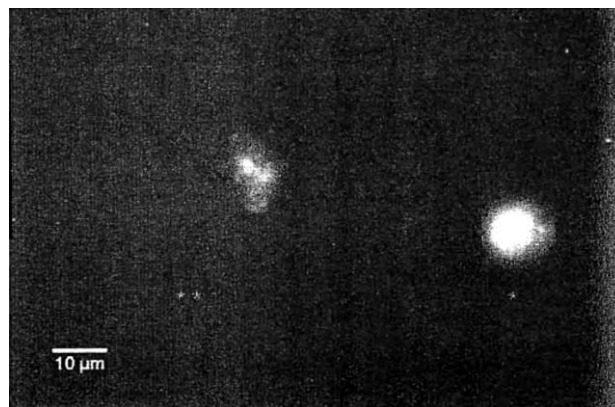

Figure 2. Photography of nuclei after electrophoresis of lysed *Chlamydomonas moewusii* cells (*): normal nucleus; (**): comet.

Under the applied experimental conditions, cell nuclei from untreated *Chlamydomonas* cells appeared as diffusely spread DNA fragments in the former nucleus area; thus, unaffected control cells form comets with a head region and with a small or without a clearly fragmented tail (Figure 2). Genotoxically damaged *Chlamydomonas* cells resulted in comets consisting of a head region and a tail of DNA fragments (Figure 2). With increasing DNA damage, fragmentation became more apparent and morphology of head region was not so clear, decreasing the fluorescence intensity of head DNA. This effect could result in the complete

disintegration of the head region, while the tail area appeared as a "cloud" of DNA fragments, culminating finally in the formation of comets without any visible area, consisting of long, extended and scattered DNA fragments. These comets shapes were difficult to recognize by eye because the weak fluorescence intensity did not provide for sufficient contrast to the slide background, and they use to receive the name of "ghost comets". This effect is probably caused by the small DNA content of the algal cells. In preliminary experiments we observed that, after 48 hours of exposure to assayed paraquat concentrations, ghosts, probably due to the strong DNA damage in cells exposed to the herbicide. In this way, we decided to analyse paraquat genotoxicity on *Chlamydomonas moewusii* only after 24 hours of exposure to these paraquat concentrations.

Damaged and even highly damaged cells were also found in control samples, and in heavily treated samples undamaged cells could be found to a certain extent. The finding of damaged cells in control samples may be due to artificial DNA damage, which can be caused by inhomogeneities in the electric field or the formation of radicals during electrophoresis (Singh et al., 1994). Further possible explanations for the heterogeneity of DNA damage in control and treated samples are variabilities in the age of cells or cell cycle status (Erbes et al., 1997; Lah et al., 2004), since synchronous cultures were not used and physiologically different cells were then present in control and in treatment cultures. However, there is a study with other microalga, *Closterium ehrenbergii,* exposed to the disinfectant triclosan, on which only occasionally were observed comets in untreated cells (Ciniglia et al., 2005).

Paraquat genotoxicity may be related with the mode of action exhibited by this herbicide. Paraquat acts on photosynthesis, as a competitive inhibitor of photosynthetic electron transport at photosystem I level (Summers, 1980; Devine et al., 1993). In this way, paraquat molecule experiments a single electron reduction and an oxidation reaction with molecular oxygen, leading to the generation of free oxygen radicals that could affect to cellular DNA (Kaloyanova and El Batawi, 1991). Reactive oxygen species (ROS) have been shown to be active genotoxic agents (Gaivao et al., 1999), since a great number of studies show that ROS are major sources of DNA damage by causing strand breaks, removal of nucleotides and a variety of modifications of the bases of the nucleotides (Cooke et al., 2003). Furthermore, paraquat is very effective in generating free radicals and cell antioxidant mechanisms, as free radical scavengers, are rapidly overwhelmed. This may explain why microalgal cultures exposed to the lowest paraquat concentration assayed showed a percentage of comets significantly higher in comparison with cultures without herbicide, only after 24 hours of exposure. Other pesticides, such as phosphamidon and dieldrin, have been reported can induce damage DNA through the generation of ROS and therefore of oxidative stress (Cicchetti and Argentin, 2003). the insecticide endosulfan has been reported to cause DNA damage that could result directly from the interaction of pesticide or its metabolites with DNA (Fairbairn et al., 1995) or through the

production of ROS (Bajpayee et al., 2006), although the precise mechanism is poorly understood (Sharma et al., 2007). In the same way, there are studies that suggest that ROS mediate herbicide-caused DNA damage (Bagchi et al., 1995; Melchiorri et al., 1998; Liu et al., 2006; González et al., 2007). the genotoxicity of paraquat has already been associated with its ability to produce superoxide anions and/or hydrogen peroxide, as it has been shown in bacteria (Moody and Hassan, 1982) and in Chinese hamster cells (Nicotera et al., 1985; Sofuni et al., 1985; Sofuni and Ishidate, 1988; Tanaka and Amano, 1989). Ribas et al. (1997/98) obtained results that indicated that paraquat is able to induce primary DNA damage using the comet assay (Ribas et al., 1995) and other gene tic endpoints.

The dose-dependent effect observed with paraquat-treated *Chlamydomonas moewusii* cells using the comet assay was also found on Euglena *gracilis* with several mutagenic compounds causing DNA damage (Aoyama et al., 2003). The alkaline comet assay was also tested on dinoflagellates *(Karenia mikimotoi* and *Alexandrium minutum)* for the study of the genotoxic effects of different pesticides such as epoxiconazole, chlorpyriphos-etbyl and endosulfan (Akcha et al., 2008).

Results obtained from different studies suggest the capability of pesticides in significantly increasing DNA fragmentation (Ribas et al., 1995; Clements et al., 1997; Sasaki et al., 1997a; Sasaki et al., 1997b; Garaj-Vrhovac and Zeljezic, 2000; Vindas et al., 2004; Akcha et al., 2008).

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