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Comet assay in phytoplankton as biomarker of genotoxic effects of environmental pollution

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Abstract:

The alkaline comet assay was tested on different microalgae: the dinoflagellates, *Karenia mikimotoi* and *Alexandrium minutum*, and the diatom, *Chaetoceros gracilis*. The microalgae were exposed during their exponential growth to the model direct genotoxicant, hydrogen peroxide (1 h, 5 and 100 µM H_2O_2). Following H_2O_2 exposure, the comet assay was validated only for *K. mikimotoi* for which genotoxicity was observed from the lowest tested concentration of 5 µM with a concentration-dependent effect. *C. gracilis* was too small in size (4 µm) to be correctly analysed. For *A. minutum*, our lysis buffer was not strong enough to digest the cellulosic thecal plates. For *K. mikimotoi*, the comet assay was thus applied for the study of the genotoxic effects of different pesticides: epoxiconazole (as Opus formulation), chlorpyriphos-ethyl (as Dursban formulation) and endosulfan at 1, 10 and 100 µg of active substance/L for 24 h. Exposure to epoxiconazole in formulation resulted in an increase in the extent of DNA strand breaks at the highest tested concentration of 100 µg/L. Genotoxicity was also observed for chlorpyriphos-ethyl in formulation from 1 µg/L ($p < 0.05$), with a significant increase at 10 µg/L. Endosulfan exposure resulted in DNA damage for *K. mikimotoi* nuclei. Genotoxicity was observed from 1 µg/L of endosulfan and was not concentration dependent.

Keywords: Phytoplankton; Pesticides; Genotoxicity; Comet assay

The phytoplankton is a model of choice for the study of the long term effects of pollutant exposure at population level. As primary producers, phytoplankton constitutes the first level of marine 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, phytoplankton offers the possibility to study the trans-generational effects of pollutant exposure. With a view to obtaining a better insight into the long term consequences of genotoxicity at population level, it appeared so valuable in a first step to test our alkaline comet assay on different microalgae. 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 et al., 2005).

For the exposure experiment, each phytoplankton strain [*Karenia mikimotoi* (GATIN95) isolated in 1995 in the bay of Brest, France; *Alexandrium minutum* (AM89BM) isolated in 1989 during a bloom in the Morlaix river, Brittany, France; *Chaetoceros gracilis* was obtained from SATMAR, Saint-Vaast-La Hougue, France] was cultivated in f/2 medium (Guillard and Ryther, 1962) at a temperature of 18°C and under controlled light (60µmol quanta/h/cm^2).
The microalgae were exposed during their exponential growth (concentration of about 50 x 10^6 cells/L) to hydrogen peroxide (1h, 5 and 100 µM), a model direct genotoxicant, and to different pesticides: epoxiconazole (as Opus® formulation form BASF), chlorpyriphos-ethyl (as Dursban® formulation from Dow AgroSciences) and endosulfan (in 0.01% DMSO) at 1, 10 and 100 µg of active substance/L for 24h exposure. The toxicity of these compounds was previously demonstrated for several strains of phytoplankton (unpublished data): for epoxiconazole and chlorpyriphos-ethyl, the effective concentration (EC50) obtained was lower when the active substance was tested in formulation.
Following exposure, the cell suspension was concentrated by centrifugation at 300g for 10 min and pellet was resuspended in 50 µL of filtrated seawater. Cell viability was assessed under the microscope by a neutral red viability test. The comet assay was then carried out as described previously in Akcha et al. (2003) by using 30 µL of the concentrated cell suspension. For each sample, two slides were prepared and the comet parameter Olive Tail Moment (OTM) of 150 cells was calculated using an image analysis system (Komet 4, Kinetic Imaging Ltd).
Following exposure, cell viability was at least 75% allowing the conduction of the comet assay. Comet data were normalised (Ln x) and analysed by an ANOVA (Statistica 6.0 Soft.), taking exposure concentration as a random factor.

Following H_2O_2 exposure, a significant dose-dependent increase in comet parameters was observed only for *K. mikimotoi* nuclei ($p < 0.005$) (Figure 1), validating the use of the comet assay for genotoxicity study in this microalgal specie. *C. gracilis* nuclei was too small in size (4 µm) to be correctly scored by our image analysis system. It is noteworthy that an application of the comet assay was already reported for another genus of this specie, *C. tenuissimus* (3-5 µm), following cadmium exposure but damage was not assessed with the image software but by counting the number of comet cells against total normal cells (Desai et al., 2006). For *A. minutum*, our lysis buffer was not strong enough to digest the cellulosic thecal plates.
Epoxiconazole in formulation was genotoxic for *K. mikimotoi* only at the highest tested concentration of 100 µg/L. DNA damage was also induced by chlorpyriphos-ethyl in formulation from the lowest tested concentration of 1 µg/L ($p<0.05$), with a significant increase at 10 µg/L. Endosulfan exposure resulted in DNA strand breaks from the concentration of 1 µg/L ($p < 0.05$), but effect did not appear to be concentration-dependent. Effective concentrations for chlorpyrifos-ethyl (Auby, personal communication) and endosulfan (Nwankwoala and Osibanjo, 1992; Leonard et al., 2001,) correspond to the higher concentrations recorded in the aquatic environment.

These results demonstrate the ability of *K. mikimotoi* to bioactivate the selected pesticides to reactive species for the DNA. Bioactivation of organic contaminants into genotoxic metabolites was already demonstrated by the SOS chromotest and by the comet assay for freshwater and marine green algae, *Selenastrum capricornutum* (Harwood et al., 1989) and *Euglena gracilis,* respectively (Aoyama et al., 2003). Despite the ability of microalgae to repair DNA strand breaks (Desai et al., 2006), genotoxic insult can have long term consequences on algal growth and diversity.
The applicability of the comet assay on phytoplankton offers valuable perspectives to study 1) the genotoxic effects of pollutants at population level, 2) the trans-generational effects of genotoxicant exposure and 3) the effects of genotoxicity on population genetics.

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Figures

Figure 1 : Effect of H_2O_2 exposure on the DNA fragmentation level of *K. mikimotoi* nuclei

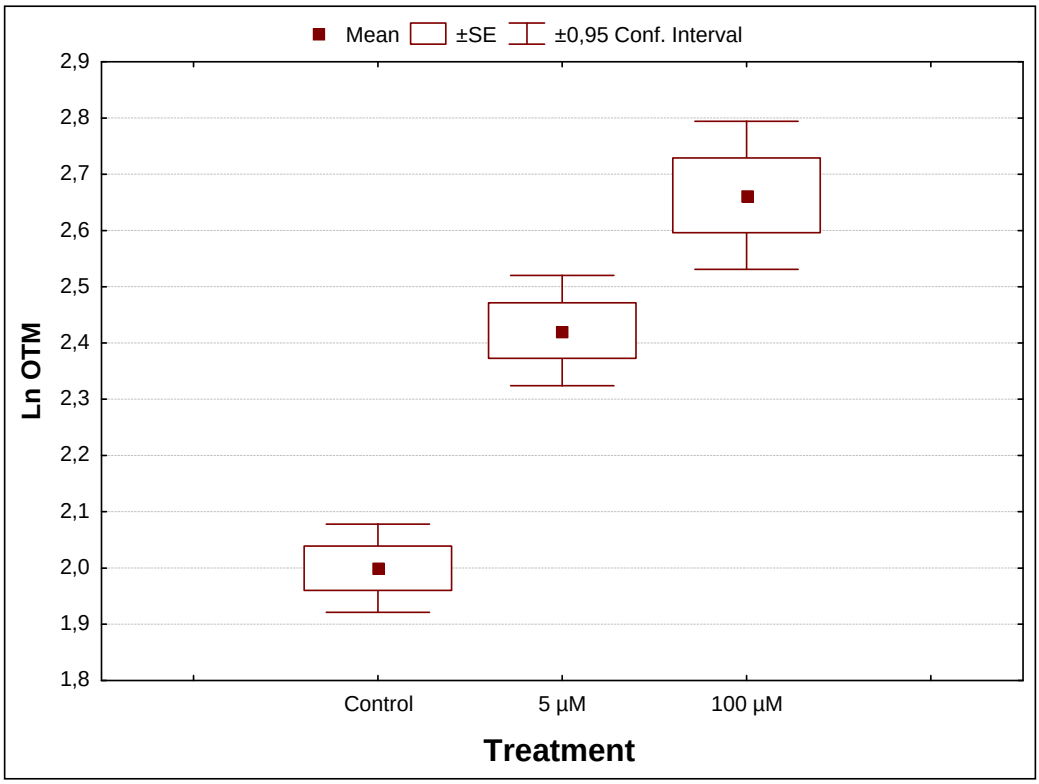

Figure 2 : Effect of epoxiconazole in formulation (A), chlorpyriphos-ethyl in formulation (B) and endosulfan (C) exposure on the extent of DNA strand breaks of *Karenia mikimotoi*