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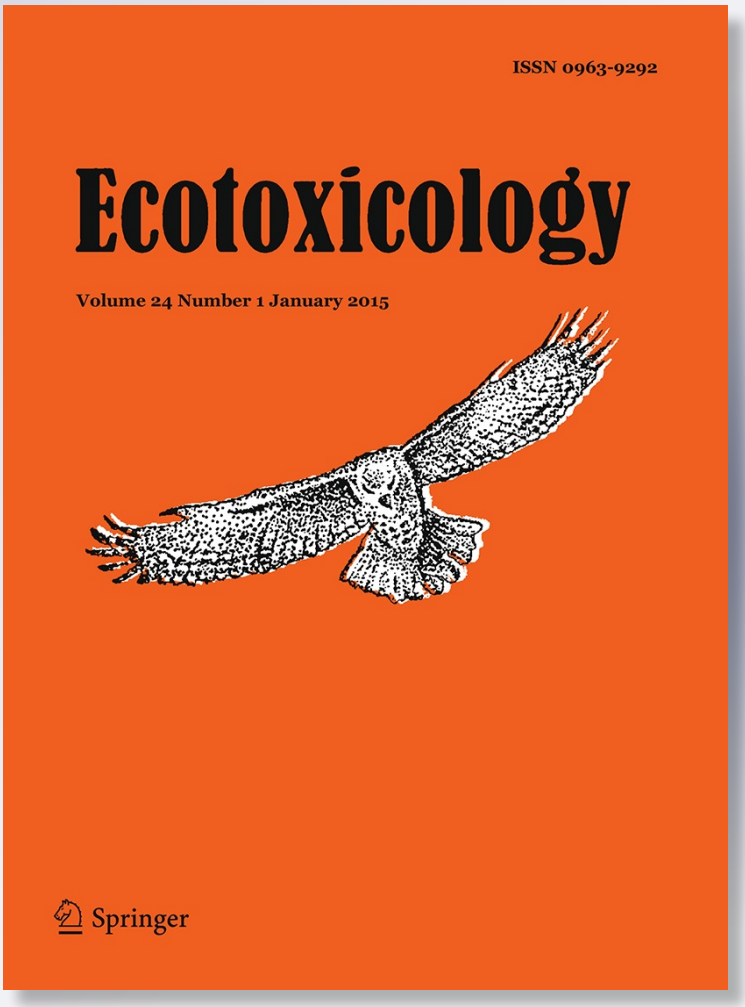

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Genotoxic effects of commercial formulations of Chlorpyrifos and Tebuconazole on green algae

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Abstract The alkaline single-cell gel electrophoresis assay (comet assay) was used for the study of the genotoxic effects of insecticide Chlorpyrifos and fungicide Tebuconazole (commercial formulations) on two freshwater green algae species, *Pseudokirchneriella subcapitata* and *Nannocloris oculata*, after 24 h of exposure. The percentage of DNA in tail of migrating nucleoids was taken as an endpoint of DNA impairment. Cell viability was measured by fluorometric detection of chlorophyll "a" in vivo and the determination of cell auto-fluorescence. Only the higher concentration of Chlorpyrifos tested resulted to affect significantly the cell viability of *P. subcapitata,* whereas cells of *N. oculata* were not affected. Tebuconazole assayed concentrations (3 and 6 mg/l) did not affect cell viability of both species. The results of comet assay on *P. subcapitata* showed that Chlorpyrifos concentration evaluated (0.8 mg/l) exerted a genotoxic effects; while for the other specie a concentration of 10 mg/l was needed. Tebuconazole was genotoxic at 3 and 6 mg/l for both species. The comet assay evidenced damage at the level of DNA simple strains molecule at pesticide concentrations were cytotoxicity was not evident, demonstrating that algae are models to take into account in ecological risk assessments for aquatic environments.

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Keywords Comet assay · Freshwater algae · Genotoxicity · Pesticides

Introduction

Pesticides are chemicals that present properties which decrees, alter or inhibit different biological processes. These toxic substances could reach aquatic environments when aerial applications are made by direct spray or by run-off from surrounding terrestrial areas. In the last years, there has been an increase of agrochemical applications in relation with crops of economic interest, e.g. soybean, and they became in one of the more frequent organic pollutants in aquatic ecosystems (Prado et al. 2009). The evaluation of risks associated with these substances discharged into the environment is of great interest and also, the development of ecotoxicological tools that allow monitoring programs included in environmental management policies.

The release of genotoxic chemicals in natural ecosystems has an impact on frequency of mutation, leading to modifications of genetic pools of wild populations. These adverse effects could alter the size of populations, affecting ecosystem sustainability (Uhl et al. 2003). Both biochemical and physiological changes as well as modifications in DNA integrity, produced by an exposure to organic pollutants, are utilized as an early signal of alarm of potential degradation of ecosystems (Vasseur and Cossu-Leguille 2003). These biomarkers allow corrective action, because if the exposition continues, a breakage in normal homeostasis processes can occur, inducing irreversible damage leading to the death of organisms (Torres et al. 2008).

According to the Argentine Crop Protection Association (CASAFE for its acronym in Spanish; CASAFE 2013) the total volume of marketing pesticides in the year 2012 was

317 million of liters. The main insecticide employed was Chlorpyrifos, with a total of 2,982,957 l, representing more than the half of all active ingredients with insecticide characteristic. Besides, fungicides as Tebuconazole, are the third more used type of pesticide, behind herbicides and insecticides. These agrochemicals products are applied on insects and fungi that affect a glyphosate tolerant transgenic variety of soybean which crops have reached to an extension of fourteen million hectares in the last year all around Argentina. They have been found in aquatic systems from 0.52 to 13 µg/l (Sáenz 2000; Sáenz et al. 2009).

Among aquatic organism, microalgae have an important role in aquatic system as they are a key component of food chains in aquatic environments due to their fundamental participation in energy conversion and ecosystem food web maintenance. So that, it is crucial have early assessment tools to evaluate toxic and genotoxic effects at the cellular level, which can lead to disturbance in structure and productivity of the algae community as these can induce direct structural changes in the rest of the ecosystem (Villem 2011). Apart from that, they have short generation time, and respond quickly to environmental changes, making them a practical model for this type of evaluations (Prado et al. 2009). Despite all mentioned above, there are few studies assessing genotoxicity on microalgae. In the literature, the most common organisms which were used as sentinel organisms in aquatic eco-genotoxicology, were fish, followed by mussel and amphibian (Yaqin 2006). There are few works in which genotoxicity is evaluated on unicellular algae (Erbes et al. 1997; Sastre et al. 2001; Aoyama et al. 2003; Desai et al. 2006; Akcha et al. 2008; Prado et al. 2009), being single-celled, they can be used as a model for assessment of DNA damage and monitoring the environmental impact of xenobiotics compounds (Cotelle and Férard 1999; Dhawan et al. 2008).

In the present study the genotoxic effects of the two most used insecticide and fungicide commercial formulation in transgenic soybean crops on common freshwater microalgae was assessed by the application of single-cell gel electrophoresis assay, also called comet assay. This method measures damage at the DNA level of living cells. There is a variety of physical agents and chemical compounds that can damage DNA of living cells. If not repaired these DNA lesions can initiate a cascade of biological consequences at the cells and finally at the population and community level (Lee and Steinert 2003; Vasseur and Cossu-Leguille 2003). Chlorpyrifos and Tebuconazole in formulation were assessed due to: (a) they are the most used in soybean crop, so its presence in freshwater systems of cultivated areas may produce deleterious effects on green algae; (b) there is evidence in several studies carried out with fish, mussels, diatoms and lymphocytes that both pesticides are genotoxic or have carcinogenic properties, fact that is supported by molecular chemical features; (c) pesticides database where genotoxic effects are reported, usually are based on studies performed with animal cells, rather than plant cells and less with microalgae, so it is important to accumulate data on responses and sensitivity of this fundamental and essential group of organism to ecosystem function. The aims of this study were: (i) contribute to the limited information about the genotoxic effect of Chlorpyrifos and Tebuconazole on microalgae, as there are scientific bases that these pesticides are genotoxic to animal cells, but few studies on microalgae; (ii) expand the knowledge about genototoxic effects of these pesticides, as they are widely used all around the world; (iii) apply microalgae in eco- genotoxicity test, specially the comet assay, as there are valuable aspects to consider, such as prediction of effects at the population level (as a consequence of cell level genotoxicty), trans generational effects (high growth rate allows several generations in short periods of time) and population recovery anomalies as a consequence of exposure to pesticides of genotoxic action. Based on this study of acute exposition, potential long term genotoxic effects cannot be estimated; these aspects will be considered in future studies.

Materials and methods

Organisms and culture conditions

Two species of green algae belonging to the Phylum Chlorophyta were used as test organisms. *Pseudokirchneriella subcapitata* strain 278/4 was obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, United Kingdom (CCAP). *Nannochloris oculata* strain LB/1998 was obtained from University of Valencia, Spain. Algae was maintained in 500 ml culture flasks containing 200 ml of mineral growth USEPA medium (USEPA 2002a). The algal culture medium was prepared by adding macro and micronutrient solutions to Milli-Q water. The pH of medium was adjusted to 7.5 (±0.1) using 0.1 N NaOH or HCl solutions as described in USEPA (USEPA 2002a). The resulting culture water had an average hardness of 200 mg/l (as CaCO3) and an average alkalinity of 50 mg/l (as CaCO3). The medium was filtered through a 0.45 µm pore diameter of cellulose nitrate membrane in a vacuum. The medium was sterilized by autoclaving (120 °C, 15 min). Algal cultures of both species were grown under continuous illumination (86 µE/m^2/s) provided by white fluorescent lamps (General Electric F15 W/54) at 24 ± 1 °C on an orbital shaker at 100 rpm. Every seventh day an aliquot of old culture was subcultivated in freshly prepared sterile medium, so cultures were maintained in exponential growth phase.

Table 1 Physico-chemicals parameters of assayed agrochemicals

	S	Log Kow	Log Koc	H
Chlorpyrifos	0.4	4.8	4.13	2.9×10^{-6}
Tebuconazole	36	3.7	3.2	1.45×10^{-10}

S water solubility (mg/l), *Kow* octanol/water partition coefficient, *Koc* sediment/water partition coefficient, *H* Henry constant (atm*m^3/mol)

Pesticides

We used commercially formulated 48 % Chlorpyrifos (*O*,*O*-diethyl -*O*-3,5,6- trichloropyridin-2-yl phosphorothioate), an organophosphorus insecticide. The other commercial formulation used was 43 % Tebuconazole (*RS*)-1-(4-Chlorophenyl)-4,4-dimethyl-3-(1H, 1,2,4-triazol-1-ylmethyl)pentan-3-ol) a triazole fungicide. Tebuconazole and Chlorpyrifos analytical standards were from Sigma (St. Louis, MO, USA). Nominal concentrations of both pesticides after 24 h of exposure were monitored by gas chromatography and mass spectrometer detector (GC–MS) according Zambonin et al. (2002) and Boyd-Boland et al. (1996), respectively. Briefly, they were extracted by solid-phase microextraction fibers (SPME) coated with 100 μm of polydimethylsiloxane (PDMS) during 1 h at 30 °C, pH 8 and 10 % NaCl (w/v). Recoveries resulted in 90–110 % and the limit of quantitation for pesticides was both 10 μg/L. Physico-chemicals parameters of active ingredients of commercial formulations are shown in Table 1 (Hornsby et al. 1996; USEPA 2012).

All other chemicals used through all the study, such as solutions of nutrient medium, buffer solution and those used along the process of the application of comet assay, were of analytical quality and purchased from commercial suppliers.

Treatment conditions

The toxicity of both pesticide commercial formulations to microalgae species used in this study has been demonstrated in previous studies (Sáenz 2000; Sáenz et al. 2009), deriving effective concentrations (EC50) lower when the active substance was tested in formulation.

Chlopyrifos and Tebuconazole (formulations) concentrations used in the present work were selected according the observed effects on growth performing dose–response algal toxicity test of 96 h of exposition following the protocol described in Sáenz et al. (2012a) (Fig. 1). The concentrations used were selected following two criteria: (1) taken into account the 96 h LOEC value derived from dose–response studies; (2) concentrations where algal growth were inhibited between 60 and 80 % respect to the control, at the end of 96 h of exposure. In this way, both algae cultures were exposure to Tebuconazole concentrations of 3 and 6 mg/l, while Chlorpyrifos assayed concentrations for *P. subcapitata* were 0.8 and 1.6 mg/l and for *N. oculata* these concentrations were 10 and 100 mg/l. Selected Chlorpyrifos concentration were so different between both algae, as dose–response toxicity test shown that *P. subcapitata* was more sensitive. Concentration range of definitive test performing on this specie was between 0.1 and 3.2 mg/l with a dilution factor of 0.5, while this range for *N. oculata* was between 0.01 and 100 mg/l applying a dilution factor of 0.1. The different sensitivity observed between these two species towards Chlorpyrifos is due to cell features, explained later. Pesticides stock solutions were prepared from commercial formulations mentioned above, by dispensing an aliquot in bidestilled and sterilized water. The nutrient USEPA medium was supplied with different pesticide concentrations from this stock solution, to achieve selected assay concentrations, to a final volume of 10 ml. Treatment pesticide concentrations were expressed as mg of active ingredient. In addition to these, cultures without pesticides were included as controls. Cultures were exposed to 100 μM hydrogen peroxide a model direct genotoxicant agent, as positive control. Microalgae cultures were exposed during their exponential growth phase of all the experiments described above during 24 h. All experiments were placed in a culture chamber at 23 °C in dark condition and were carried out in duplicate.

Effects of pesticides exposure on cell viability

Algal cell viability was systematically measured prior to each experiment to assess the potential cytotoxic effects of both pesticide exposure concentrations. This was an important task in order to legitimate the use of the alkaline comet assay as a reliable genotoxicity assessor. Viability was assessed by means of two different tests:

Measure of chlorophyll "a" in vivo. Chlorophyll fluorescence is considering a probe of photosynthesis in vivo, yet the emission of fluorescence is related directly with light absorbed by chlorophyll molecules and means a correct function of photosystem II (PSII). PSII is indicative of the whole rate of photosynthesis and is considered the most vulnerable part of the photosynthetic apparatus when a stressor is acting. Measure of chlorophyll "a" in vivo was done with a Turner TD 700 fluorometer (USA) using an excitation wavelength of 420 nm and measuring the emission at 630 nm. Correlation between fluorometric unit and actual chlorophyll concentrations were made using a serial of five standard of known concentrations prepared from pure chlorophyll "a" extract. A cuvette was filled with ten milliliters of controls and treated cultures and pigment concentrations were recorded. Algal cells of exposed cultures

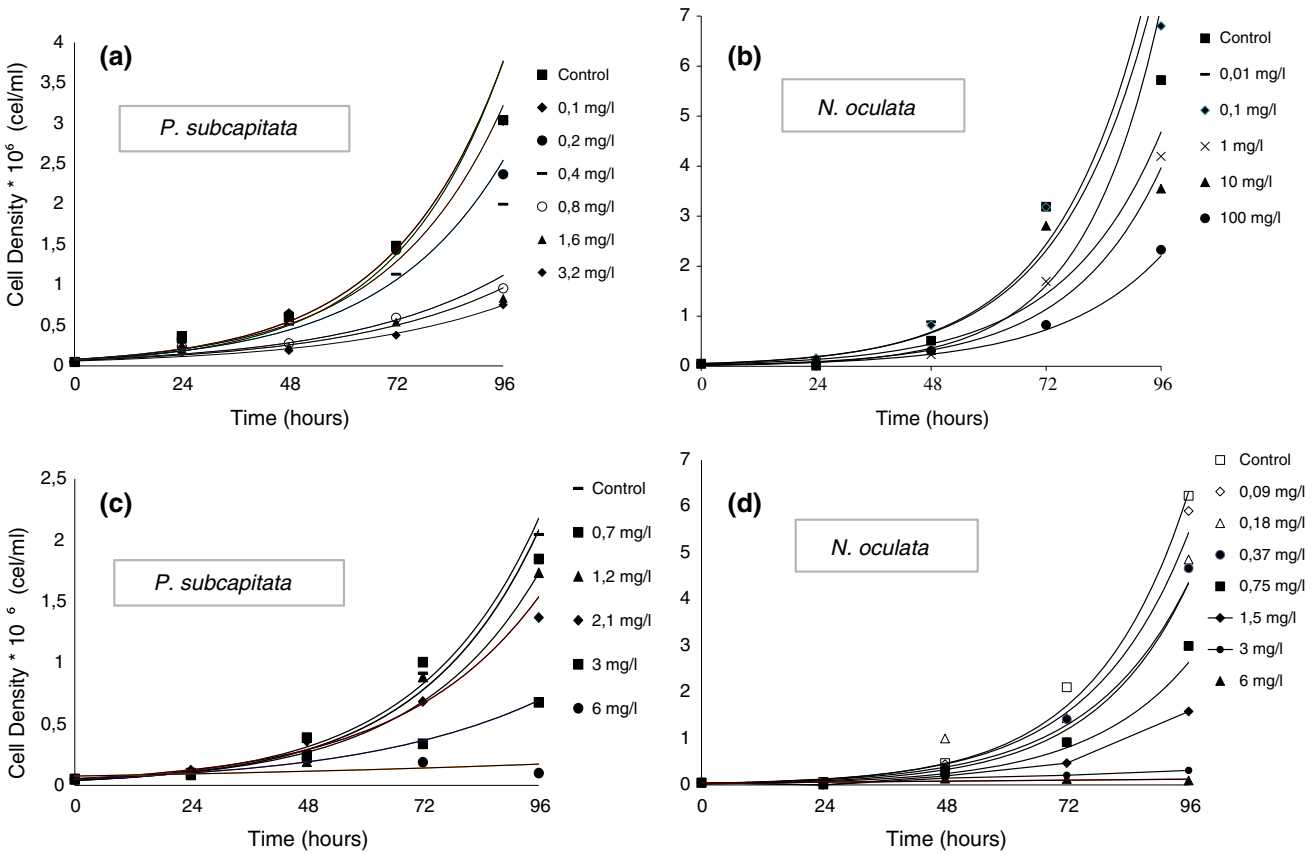

Fig. 1 Growth curves of green algae treated with different concentrations of Chlorpyrifos (**a**, **b**) and Tebuconazole (**c**, **d**)

were considered alive when chlorophyll concentration fluorescence of treated cultures was equal or up to a 20 % less than those of controls (Martinez 2013). Determinations were recorded in µg chlorophyll "a"/ml. Results were expressed as a percentage (%) of pigment respect to the control.

Measurement of chlorophyll auto-fluorescence. Emitted (fluorescent) light as a consequence of illuminating algal cells by a mercury lamp in a Nikon Eclipse 600 microscope equipped with an epifluorescence device and respective filters was used to measure auto-fluorescence of algal cells. For this procedure treated and control cultures were centrifuged, then re-suspended in phosphate buffered saline (PBS), and mounted on a slide. One hundred to one hundred and fifty cells were counted in control and treated samples. Those cultures having a number of cells with fluorescence emission not less than 80 % compared to control were considered viable (Martinez 2013).

Comet assay

The alkaline single-cell gel electrophoresis or comet assay was applied to detect the DNA damage induced after 24 h of exposure to both pesticides in formulation on green microalgae *P. subcapitata* and *N. oculata*. The comet assay protocol used was according the method applied in Di Marzio et al. (2005) with a modification of algal cells, related to lysing times (Martinez 2013).

Slide preparation

At the end of the exposure period, cells from each culture (treated and controls) were centrifuged (2000 rpm, 5 min), the supernatant was discarded and pellet re-suspended in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Cell suspensions were prepared in order to achieve a final density of 7.69×10^6 cells/ml in the case of *P. subcapitata* and 14.93×10^6 cells/ml in the case of *N. oculata.* Two replicate slides were prepared for each treatment culture and negative control. The procedure was as follows: 500 µl of the cell suspension were mixed with 500 µl of a 1 % low-melting point agarose solution in PBS. 80 µl of this mixture were deposited on a slide to form the middle layer, sandwiched between a bottom layer of 0.5 % normal-melting point agarose solution in PBS and a top layer of 0.5 % low-melting agarose solution. The slides were immediately placed on ice in the dark for ten minutes to allow the agarose to solidify.

Slide processing

Slides with coverslip removed were immersed in freshly prepared cold lysis solution [2.5 M NaCl, 100 mM $Na_2EDTA.2H_2O$, 10 mM Tris (pH 10), 1 % *N*-laurylsarcosinate, 1 % Triton X-100, and 10 % dimethylsulfoxide (DMSO)] for 1.5 h. After a quick wash in electrophoresis buffer they were placed in an horizontal electrophoresis tank and covered with freshly prepared electrophoresis buffer (300 mM NaOH, 1 mM $Na_2EDTA{\cdot}2H_2$, pH 13.5) for 20 min at room temperature to allow DNA unwinding. Electrophoresis was conducted with the same buffer for 30 min at 20 V and 300 mA. At the end of electrophoresis, slides were washed by immersion in neutralization buffer (0.4 M Tris, pH 7.5) for 10 min and then stained with 20 µl of 2 µg/ml ethydium bromide solution.

Slides reading and statistical analysis

Stained slides were observed at 1000× magnification using a Nikon Eclipse 600 microscope equipped with epi-fluorescence light path system with a mercury lamp. Images were digitized using a magnification of 1000× with a basal low-fluoresce immersion oil. Comets analysis was made using CASP program (Casplab.com) measuring DNA migration parameters, such as mean, standard deviation, median and the 3rd quartile. % Tail DNA comet assay parameter was choosen as it is not measured in arbitrary units, being more meaningful and advisable for regulatory purposes and for inter-laboratory comparisons (Kumarravel and Jha 2006). A square root transformation was applied to Tail DNA (%) data to stabilize the variances and approximate a normal distribution. One hundred and fifty nucleoids were evaluated from each duplicate slide, per each assayed concentration, according to Tice et al. (2000). Measured values were shown by a box, with mean values represented by closed squares. The whiskers marked 0.95 confidence intervals. Differences observed were evaluated by one-way ANOVA according Zar (2010) and Dunnett's multiple comparison test was used to determine significant differences among treatment groups and control groups (not treated). These analyses were made by Statistica V8 program.

Results and discussion

Cultures exposed to Chlorpyrifos in formulation

Pseudokirchneriella subcapitata

After 24 h of exposure to selected concentrations (0.8 and 1.6 mg/l) of the commercial formulated insecticide, viability was determined. The results of chlorophyll "a" in vivo content of algal cells exposed to Chlorpyrifos evaluated fluorometrically expressed as percentage are shown in Table 2. Cells exposed for 24 h to a concentration of 0.8 mg/l did not show a significant difference in fluorescence compared to the control, whereas those exposed to 1.6 mg/l shown a significant decrease in chlorophyll content of over 68 %. This result allowed conclude, that concentrations of 1.6 mg/l of Chlorpyrifos in formulation resulted cytotoxic to *P. subcapitata* cells. Auto-fluorescence of algal cells was in accordance with the above results, as cells count at 0.8 mg/l showed only a decrease in a 10–20 % of cells emitting auto-fluorescence, determining that such concentration did not result cytotoxic for this specie. Nevertheless, algal cells exposed to the highest concentration of 1.6 mg/l did not emit auto-fluorescence, in accordance with chlorophyll "a" content measures.

Table 2 Cell viability (percentage of chlorophyll "a" in vivo concentration) of algal species after 24 h of exposure to selected pesticides concentrations

Specie	Control	Chlorpyrifos (mg/l)				Tebuconazole (mg/l)	
		0.8	1.6	10	100	3	6
P. subcapitata	100	96	32	n.d.	n.d.	100	100
N. oculata	100	n.d.	n.d.	100	87	100	100

n.d. not determined

The above results allowed the conduction of comet assay on algal cells exposed to not citotoxic concentration (only 0.8 mg/l). DNA damage of treated cells and control cultures (negative and positive) are illustrated in Fig. 2a. Chlorpyrifos in formulation was genotoxic to this algal specie at a concentration of 0.8 mg/l, as significant differences were found compared with control cells. At this insecticide concentration mean values of % tail DNA was 21.9, Besides, 90 % of the cells had 51 % of DNA in tail, indicating a fragmentation of nuclei. In this case the mean value of tail DNA was 28.6, while 90 % of cells had a % of tail DNA of 81. Control cells showed a mean value of tail DNA of 2.1 % while 90 % of cells had only 8.6 %, indicating a scarce fragmentation of nuclei.

Nannochloris oculata

The viability of exposed algal cells was measured after 24 h of exposure to selected concentrations of 10 and 100 mg/l of Chlorpyrifos in formulation. The results of "in vivo" chlorophyll "a" content of algae cultures are shown in Table 2. *N. oculata* cells exposed to both concentrations of the insecticide had chlorophyll "a" in vivo concentrations not significantly different from not exposed cells (control)

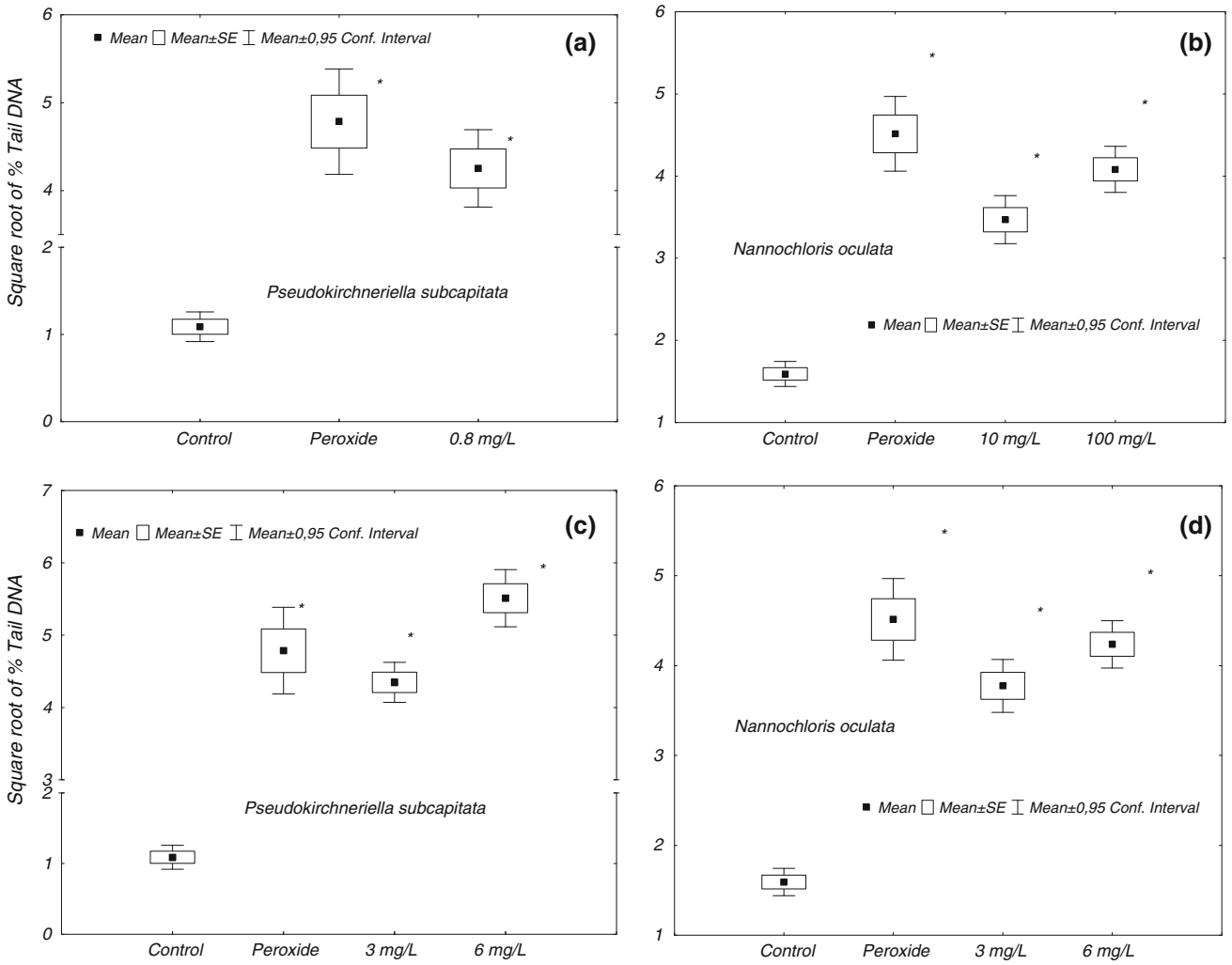

Fig. 2 Effect of Chlorpyrifos on DNA integrity of *P. subcapitata* (**a**), *N. oculata* (**b**) and of Tebuconazole on *P. subcapitata* (**c**), *N. oculata* (**d**). Exposure to the direct genotoxicant hydrogen peroxide (Peroxide) (100 μM). *Significantly different at $p < 0.05$ (One-way ANOVA–Dunnett test)

($p > 0.05$). These determinations indicated that such concentrations did not result cytotoxic for this specie, being in concordance with the evaluation of auto-fluorescence that showed, for both concentrations, similar proportions of emitting cells than control. The comet assay was conducted with algae exposed to both concentrations of insecticide formulation. Results obtained in the assay showed that both concentrations of 10 and 100 mg/l induced DNA damage in cells of *N. oculata* after 24 h of exposition (Fig. 2b). At these concentrations significantly differences were found between exposed algal cells and control ones ($p < 0.05$). Mean values of tail DNA were 15 and 19 %, respectively, while 90 % of nuclei had a percentage of DNA present in the comet tail (% tail DNA) of 33 and 38, respectively. Treatment concentrations showed a dose-dependent DNA damage, as it was increased as insecticide concentration increase. The measured Chlorpyrifos concentrations after 24 h did not differ more than 10 % with respect to the nominal values.

Genotoxicity of Chlorpyrifos

In the present study, Chlorpyrifos formulation produced a genotoxic effect on both species. The lower concentration of this insecticide needed to exert a genotoxic effect over *N. oculata* (10 mg/l) cells were higher than for *P. subcapitata* (0.8 mg/l). As mentioned above, the different sensitivity observed between these two species towards Chlorpyrifos is due to cell features. Cells of *N oculata* are surrounded by a layer of mucilage involving the cells, that would act as an adherent surface for component of the formulation or the active ingredient itself, since as indicated in Table 1, Koc of Chlorpyrifos is 4.13 which means that has tendency to stick to organic matrices. Bioavailability would be reduced, not

being incorporated into the cell, resulting in less toxic effects. This layer is absent in *P. subcapitata* cells, so the substances are incorporated into the cells, producing more severe toxicological effects on cell division and growth (Schubert 2003).

There are few studies about genotoxic effects of Chlorpyrifos on algae. Akcha et al. (2008) evaluated the genotoxicity of different pesticides, among them Chlorpyrifos (in commercial formulation), on the dinoflagellate algae *Karenia mikimotoi*. They found that a concentration of 1 μg/l was enough for caused DNA damage. There is evidence that Chlorpyrifos produce genetic damage in vivo and in vitro different biological models, using different approaches such as, chromosome aberrations, micronuclei, mutagens and even comet assays. Rahman et al. (2002) tested the ability to induce in vivo genotoxic effects of Chlorpyrifos, orally administered, on mice leukocytes. They found that 24 h after treatment with 0.28 mg/kg of b.w. caused a significant increase in mean comet tail length indicating DNA damage, which was dose related. Ali et al. (2008) measured the genotoxic effect of this insecticide on lymphocytes and gill cells of the freshwater fish *Channa punctatus*. After 96 h of exposure the authors found DNA damage, as a percentage of tail DNA, from concentrations of 203 μg/l. Mehta et al. (2008) reported that Chlorpyrifos cause dose-dependent increase in DNA damage in the liver and brain of exposed rats. Studies conducted on human leukocytes shown that 25 mg/l of Chlorpyrifos provoked duplication of comets length respect to control cells (Vindas et al. 2004).

Cultures exposed to Tebuconazole

Pseudokirchneriella subcapitata

Applying the same methods than with the previous pesticide, viability of algae cells was determined. The results of chlorophyll "a" content of cells exposed to Tebuconazole evaluated fluorometrically are shown in Table 2. None of the two concentrations evaluated exerted effects in chlorophyll "a" content as both did not differ significantly from the control. These determinations indicated that such concentrations of Tebuconazole in formulation did not result cytotoxic for the cells of this specie. This was in accordance with the proportion of cells with auto-fluorescence of exposed cultures and those considered controls. Statistical analysis of comet assay showed that there was a significant difference between cells exposed to 3 and 6 mg/l and control ($p < 0.05$) (Fig. 2c). This means that the fungicide at assayed concentration caused a genotoxic effect on the cells. Cells exposed to 3 mg/l resulted in mean values of % tail DNA of 24, while 90 percentile had a % tail DNA value of 54. At higher concentration of 6 mg/l, these values were 35 and 66, showing increasing damage when increasing concentration.

Nannochloris oculata

Cell viability data taken by fluorometric determinations of control and cultures exposed to 3 and 6 mg/l of Tebuconazole in formulation were similar between them, indicating that fungicide did not exert cytotoxic action (Table 2). The proportion of cells (control and exposed) with auto-fluorescence were similar, agreeing with previous results.

Both concentrations resulted genotoxic to this specie, as statistical analyses shown differences from control cells (Fig. 2d). Cells exposed to 3 mg/l showed a mean % tail DNA of 18, while 90 % of cells had a % tail DNA of 42, generating a significant difference from control ones ($p < 0.05$). At 6 mg/l these values were greater, (22 and 47) yielding a significant difference from not exposed cells ($p < 0.05$). Anyway both assay concentrations of Tebuconazole in formulation were more harmful to *P. subcapitata*, as a % of tail DNA of treated cells were higher than those found in *N. oculata*. The measured Tebuconazole concentrations after 24 h did not differ more than 10 % with respect to the nominal values.

Cells of both species exposed to hydrogen peroxide (positive control) presented significant genotoxic damage, the effect was systematically observed at 100 μM, yielding a mean value of % tail DNA of 28 for *P. subcapitata* and 26 for *N. oculata*. These results justify its use as a positive control for the comet assay.

Genotoxicity of Tebuconazole

Tebuconazole in formulation exerted genotoxic action on both species in both exposition concentrations, being a bit more aggressive to *P. subcapitata*. A study had observed a drastic reduction in carbohydrates, proteins and sugar levels in three species of cyanobacteria after being in the presence of this fungicide (Kumar et al. 2012). These authors propose that the reduced algal growth observed could be caused by a decrease in levels of enzymes and in the synthesis of structural proteins. The present work showed that the fungicide employed concentrations provoked damage in genetic material which eventually could trigger imbalances in normal amount of different proteins required by the organisms. Akcha et al. (2008) evaluated the genotoxicity of Epiconazole (also a triazole fungicide) in the commercial formulation on the microalga *K. mikimotoi*. The authors found that this fungicide increased the DNA strand breaks at the highest tested concentration of 100 μg/l.

Chemicals striking the integrity of cellular DNA are potentially mutagenic and carcinogenic agents. Tebuconazole

is listed as a possible carcinogen rated as C (possible carcinogen) according to USEPA (USEPA 2002b) and USEPA (USEPA 2010), so the effects observed on microalgae is an important evidence of genotoxic potential. The production of damage on DNA strands correlates with the mutagenic and carcinogenic properties of environmental pollutants with diverse structures. Several studies have demonstrated that fungicide Imazali, whose chemical structure and mode of action is similar to Tebuconazole, produced genotoxic damage on DNA strands when human lymphocytes were exposed to 75 mg/l (Vindas, et al. 2004). Others triazole fungicides such as, Epoxiconazole, Cyproconazole, Fluconazole, Propioconazole, Triadimenol were responsible of genotoxic damage in hepatic cells, liver cells and lymphocytes (Fucic et al. 2008; Goetz and Dix 2009; Nesnow et al. 2011; OEHHA 2011).

Genotoxicity of pesticides on algae

Ruptures than occur in DNA molecules can be produced by direct action of Chlorpyrifos and Tebuconazole on genetic material, consisting in the chemical modification of DNA. This modification produces alterations on nitrogenous bases, either adducts or abasic sites of the molecule. Most of the substances considered genotoxic or carcinogenic are highly electrophilic or can be activated to electrophilic metabolites once inside the cell, by xenobiotic detoxification mechanisms of the living organisms. Electrophilic molecules are capable of covalently bonding to biological molecules. Phosphoryl group of Chlorpyrifos is an electrophilic site that can react with DNA molecule. Alkyl group has the capacity of reaction with nucleophilic centers of DNA molecules, as nitrogen 7 of the guanine. The alkyl group and the triazole polar ring of Tebuconazole facilitate the binding to the DNA molecule altering and reacting with the molecule. In this way Chlorpyrifos and Tebuconazole could be acting as alkylating agents, reacting with highly nucleophilic centers of nitrogen atoms of DNA or with oxygen atoms. The alkylation of oxygen is related to mutagenic effects while of nitrogen is related to citotoxicity. Phosphoryl polarity (in Chlorpyrifos) and triazole ring (in Tebuconazole) could favor the binding with DNA, which would lead to adduct formation that would be translated in breakage when comet assay is performed. Furthermore there is evidence that Chlorpyrifos inhibits the synthesis of DNA (Qiao et al. 2003).

There are few studies about the genotoxic damage on algae species caused by the action of other pesticides. Debenest et al. (2008) found that an exposure of a freshwater benthic diatom community to the herbicide maleic hydrazine, elicited nucleus alterations observed as: abnormal nucleus location, micronucleus, multinuclear cell or disruption of the nuclear membrane. Apart from that, an induction of abnormal frustules was observed. Their results suggest that the alterations could be related to the effects of maleic hydrazine on the synthesis of the proteins involved in frustule formation or in the regulation of the cytoskeleton of the diatom cells. In another study it was demonstrated, by application of the comet assay, that Triclosan exerted significant genotoxic effects on *Closterium ehrenbergii* at 0.25 mg/l; and in higher concentrations caused irreversible alterations in DNA strands (Ciniglia et al. 2005). Prado et al. (2009) demonstrated that the herbicide Paraquat induce significant DNA damage in cells of the freshwater microalga *Chlamydomonas moewusii* after 24 h of exposure to 0.05 µM. Akcha et al. (2008) also evaluated the genotoxicity of the active substance of insecticide endosulfan on the microalga *K. mikimotoi* observing that a concentration of 1 µg/L was enough to caused DNA damage.

Respect to environmental concentrations of pesticides used in agricultural practices, it is worth to consider that due to the large extensions of land to be treated with agrochemicals substances, aerial applications are the practice most frequently chosen. In these situations agrochemicals are sprayed directly on streams and rivers or they entry to aquatic systems by spray drift. Depending on rainfall intensity, the amount of water leaving the fields via surface runoff can be important; heavy rain occurs during pampasic summer which is the season where transgenic soybean is grown. As a result of these practices, high concentrations of agrochemicals used in soybean crops can be found in aquatic systems, causing genotoxic effects on microalgae reported in this study, besides others effects on photosynthesis, growth and oxidative stress enzyme just reported in Sáenz et al. 2009 and Sáenz et al. 2012a, b.

Conclusion

An induction of genotoxic damage in *P. subcapitata* and *N. oculata* by both Chlorpyrifos and Tebuconazole in formulation was evidenced by the application of the comet assay method. The differential sensitivity of the two green algae was compared. In cells of evaluated species, the comet assay evidenced damage at the level of simple strands of the DNA molecule in concentrations were cytotocicity was not evident, demonstrating that are models to take into account in ecological risk assessments for aquatic environments.

At the moment of making an assessment of the genomic integrity, it is important to have a reliable methodology since it has been proposed that an increase in its instability plays a critical role in the decrease of aptitude thus in population permanence, not only in the laboratory but also

in field conditions (Jha 2008). If the modifications are not repaired or it is not efficiently repaired, DNA damage will bring effects in subsequent development, deteriorating algae population dynamics as well as the reproductive success of exposed organisms. This would lead to adverse effects in the long term survival of populations and therefore affecting ecosystems quality.

The Comet assay could reflex an important damage in DNA integrity, so it is probable that this leads ripple effects on higher levels of biological organization. Hence if what is sought is predicted potential ecological consequences, it is a methodology to take into account. We concluded that it can be considered that the comet assay provides an economic, sensible and fast method for observation of DNA breaks in algae, which is ideal as a not specific genotoxical biomarker and could be an important aspect of environmental genotoxicity monitoring in surface waters, especially on this fundamental group of organisms.

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Conflict of interest The authors declare that they have no conflict of interest.

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