Ecotoxicology and Environmental Safety 120 (2015) 334–341

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Effects of chlorpyrifos on the growth and ultrastructure of green algae, *Ankistrodesmus gracilis*

Viviana Asselborn [a], Carolina Fernández [b], Yolanda Zalocar [c], Elisa R. Parodi [a,b,*]

[a] Laboratorio de Ecología Acuática, Depto. Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, B8000ICL Bahía Blanca, Argentina
[b] CONICET-BB-IADO (Instituto Argentino de Oceanografía), CC 804, B8000FWB Bahía Blanca, Argentina
[c] CONICET-CECOAL (Centro de Ecología Aplicada del Litoral), CC 291, 3400 Corrientes, Argentina

ARTICLE INFO

Article history:
Received 6 August 2014
Received in revised form
8 June 2015
Accepted 9 June 2015

Keywords:
Pesticide
Microalgae
Toxicity
Ultrastructure

ABSTRACT

The effect of the organophosphorus insecticide chlorpyrifos on the growth, biovolume, and ultrastructure of the green microalga *Ankistrodesmus gracilis* was evaluated. Concentrations of 9.37, 18.75, 37.5, 75 and 150 mg L^{-1} of chlorpyrifos were assayed along with a control culture. At the end of the bioassay the ultrastructure of algal cells from control culture and from cultures exposed to 37.5 and 150 mg L^{-1} was observed under transmission (TEM) and scanning electron microscopy (SEM). After 24 and 48 h, treatments with 75 and 150 mg L^{-1} inhibited the growth of *A. gracilis*; whereas after 72 and 96 h, all the treatments except at 9.37 mg L^{-1} significantly affected the algae growth. The effective concentration 50 (EC_{50}) after 96 h was 22.44 mg L^{-1} of chlorpyrifos. After the exposure to the insecticide, an increase in the biovolume was observed, with a larger increase in cells exposed to 75 and 150 mg L^{-1}. Radical changes were observed in the ultrastructure of cells exposed to chlorpyrifos. The insecticide affected the cell shape and the distribution of the crests in the wall. At 37.5 mg L^{-1} electodense bodies were observed along with an increase in the size and number of starch granules. At 150 mg L^{-1} such bodies occupied almost the whole cytoplasm together with lipids and remains of thylakoids. Autospores formation occurred normally at 37.5 mg L^{-1} while at 150 mg L^{-1} karyokinesis occurred, but cell-separation-phase was inhibited. The present study demonstrates that the exposure of phytoplankton to the insecticide chlorpyrifos leads to effects observed at both cellular and population level.

1. Introduction

There is widespread application of pesticides to control pests and diseases attacking agricultural products. Today, more than 10,000 chemicals are used for industrial and agricultural purposes (Katsumata et al., 2006). After organochlorine insecticides removal from usage, organophosphorus insecticides have become the most widely used compounds available. Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is one of the several organophosphorus compounds developed in the 1960s to replace persistent organochlorine pesticides. It is the most intensively used organophosphorus insecticide in agriculture (Larson et al., 1997) and is prevalent in urban insecticide applications (Schiff et al., 2002).

Chlorpyrifos is commonly used to control foliage and soil-borne insect pests on a variety of food and feed crops. Urban uses of chlorpyrifos include landscape maintenance, structural pest control, dormant sprays, and pet products (Solomon et al., 2014). It is one of the most widely used organophosphorus insecticides in agricultural activities in Argentina (CASAFE, 2009).

As other organophosphorus insecticides, the primary site of action of chlorpyrifos in animals is the acetylcholinesterase enzyme, which is inactivated by phosphorylation, thereby interfering with normal cholinergic nerve transmissions (USEPA, 1986).

Because of their chemical instability, organophosphorus pesticides such as chlorpyrifos are considered to be non-persistent in the environment and they do not tend to accumulate in the tissues (Lacorte et al., 1995). However, experimental research with 14C-labelled chlorpyrifos has shown that this pesticide may persist for relatively long periods in lagoon sediments, allowing the build-up of sedimentary reservoirs (Carvalho et al., 2002).

Organophosphorus insecticides can contaminate surface waters through unintentional drift of aerial spraying in agricultural usage, watershed drainage or accidental spillage (Sabater and Carrasco, 2001a). Freshwater species can thus be exposed to organophosphorus insecticide concentrations which range from lethal to

* Corresponding author at: CONICET-BB-IADO (Instituto Argentino de Oceanografía), CC 804, B8000FWB Bahía Blanca, Argentina. Fax: +54 291 486 1112.
E-mail address: elipapers@criba.edu.ar (E.R. Parodi).

http://dx.doi.org/10.1016/j.ecoenv.2015.06.015
0147-6513/

sublethal (Streit and Kuhn, 1994).

Few reports are available on the effects of chlorpyrifos on nontarget aquatic organisms. Some authors have demonstrated adverse effects on aquatic invertebrates and fish. Varó et al. 1998 pointed out EC_{50} values ranging from 0.95 to 18 mg L^{-1} of chlorpyrifos in several *Artemia* species. Besides, in toxicity studies using the fish species *Aequidens portalegrensis* as test organism, low concentrations of chlorpyrifos (140, 250 and 450 μg L^{-1}) produced a 100% of mortality (Domitrovic, 1997). Among non-target species, algae need special attention considering their ecological position at the base of most aquatic food webs along with their essential roles in nutrient cycling and oxygen production. In aquatic ecosystems microalgae may accumulate pesticides from the medium and so play a key role in the transport of organic contaminants through the food chain to higher trophic levels (Wang and Wang, 2005).

It is well known that microalgae are sensitive to a large scale of contaminants including heavy metals (Atici et al., 2008; Mayer-Pinto et al., 2011; Wong and Chang, 1988), herbicides (Anton et al., 1993; Moro et al., 2012; Rioboo et al., 2002; Sáenz et al., 1997, 1993; Sabater et al., 2002; Vendrell et al., 2009), insecticides (Gómez de Barreda Ferraz et al., 2004; Sabater and Carrasco, 2001a, 2001b; Wendt-Rasch et al., 2003) and industrial effluents (Tukaj et al., 1998; Walsh and Alexander, 1980). Even though studies on the effects of chlorpyrifos on microalgae are scarce, adverse effects have been reported both at cellular and population level. Important alterations in the morphology and growth of *Selenastrum capricornutum* exposed to different concentrations of chlorpyrifos were observed by Asselborn et al. (2000), whereas Stratton (1987) found that low concentrations of this insecticide produced a decrease in the specific diversity of diatoms. Moreover, Martinez et al. (2015) found evidences of damage at DNA simple strains molecule level in two green algae at low chlorpyrifos concentration.

The aim of this study was to evaluate the effects of different concentrations of the organophosphorus insecticide chlorpyrifos on the growth, biovolume and ultrastructure of the green microalga *Ankistrodesmus gracilis* (Reinsch) Koršikov.

2. Materials and methods

2.1. Species test and toxicity bioassays

Cultures of the unicellular freshwater alga *A. gracilis* were obtained from Dr. Armando Vieira's culture collection at the Universidad Federal de São Carlos (SP), in Brazil and kept in Bold's Basic Medium (Stein, 1973).

The stock culture was prepared from these strains. It was incubated during 7 days at a temperature of 24 ± 2 °C under continuous illumination (2300 lx) in order to obtain a culture in an early phase of exponential growth. Three daily manual swirling agitations were performed. The bioassay was carried out according to the OECD guideline 201 (OECD, 2006). A commercially formulated 10.5% chlorpyrifos (Dursban 10.5 Lee, Dow Agrosciences Argentina S.A.) was used. The formula was diluted in sterile distilled water and added to sterile Bold's Basic medium in order to obtain the following nominal concentrations of the active ingredient (a.i.): 9.37, 18.75, 37.5, 75 and 150 mg L^{-1}; these concentrations were selected through a previous assay in which the microalgae was exposed to a wider range of chlorpyrifos concentration. An initial inoculum of *A. gracilis* cells was added to each dilution in order to obtain an initial concentration of 5.10^4 cells mL^{-1}. A control treatment was incubated in the same culture medium without chlorpyrifos. All assays were carried out in duplicate, without medium replacement, in 250 mL glass Erlenmeyer flasks containing 100 mL of test medium. The flasks with the treatments were placed in a culture room at 24 ± 2 °C under continuous illumination (2300 lx). Three daily manual agitations were performed.

To evaluate the effects of chlorpyrifos on the population growth, the number of microalgae in each treatment was determined by direct counting in Neubauer chamber every 24 h during the entire assay (96 h). To evaluate the effects of chlorpyrifos on cellular volume, linear dimensions; i.e., large diameter of ellipse, small diameter of ellipse and diameter of cell, of thirty cells from each treatment were measured every 24 h under a light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and the biovolume was calculated according to Sun and Liu (2003). Simultaneously, the shape, color and presence of granules in the microalgae were observed.

Exponential growth rate (r, h^{-1}) was calculated using the following equation (Fogg, 1975):

$$r = \frac{\ln N_1 - \ln N_0}{t}$$

where N_1 is the cell concentration (cells mL^{-1}) at the end of the assay, N_0 is the initial cell concentration (cells mL^{-1}) and t is the exposure time in hours.

Generation time (G_t, h); i.e., time during which a population is duplicated, was determined by means of the following equation (Reynolds, 1984):

$$G_t = \frac{\ln 2}{r}$$

The percent inhibition of algal growth rate was calculated with respect to controls according to the following equation (USEPA, 1989):

$$\%I = \frac{T - C}{C} \times 100$$

where T is the average specific growth rate (r) for the treatment replicate and C is the mean value for average specific growth rate in the control.

Effective concentration 50 values (EC_{50}); i.e., concentration which produces adverse effects on 50% of the population, and confidence intervals (95%) for each exposure time were calculated by means of a Probit analysis (Finney, 1971) of the percent inhibition of algal growth rate (%I) using the Infostat software package (2008).

One way analysis of variance (ANOVA) was used to evaluate whether there were significant differences between microalgae population growth (cell concentration, r and G_t) of the different treatments, when statistical differences among values were detected, Tukey´s multiple comparisons pairwise test was applied. The non-parametric Kruskal–Wallis test was used to assess differences in microalgal biovolume between treatments since data did not attend criteria for parametric methods (Zar, 2009).

2.2. Electron microscopy techniques

For transmission electron microscopy (TEM), we followed the method described by Cáceres (1995) while for scanning electron microscopy (SEM), we followed to Boltovskoy (1995). At the end of the bioassay, the control culture and the 37.5 and 150 mg L^{-1} a.i. treatment were centrifuged (all replicates pooled together) at 4000 rpm for 5 min and the resulting pellet was fixed.

Material for TEM was fixed at 4 °C in 2.5% glutaraldehyde (Sigma-Aldrich) and postfixed in 1% OsO_4 (Sigma-Aldrich) using filtered culture medium as fixative vehicle. The material was subsequently dehydrated in an acetone series from 10% to absolute acetone. It was then embedded drop by drop in Spurr's low-

viscosity resin (Spurr, 1969) and flat-embedded between glass slides coated with dry Teflon (Reymond and Pickett-Heaps, 1982). Sections were cut with a Diatome 2.1 mm diamond knife (Diatome Ltd., Bienne, Switzerland), mounted on Formvar-coated grids (Polysciences, Inc., Warrington, PA) and stained with uranyl acetate and lead citrate. They were examined under a Jeol 100 CX-II electron microscope (Jeol Ltd., Akishima, Tokyo, Japan) coupled with a Gatan Erlangshen 785 CCD (Gatan Inc. Warrendale, PA, US) digital camera at CCT-Bahia Blanca.

For SEM, cells were fixed in 2.5% glutaraldehyde (Sigma-Aldrich) in filtered culture medium at 4 °C and subsequently settled on Melinex film coated with 0.5% poly-D-lysine (Sigma-Aldrich). Cells were dehydrated in an acetone (Sigma-Aldrich) series and dried in a critical point dryer. Samples were coated with gold in a sputter coater, and observed under a Jeol JSM 35 CF scanning electron microscope (Jeol Ltd., Akishima, Tokyo, Japan) coupled with a MAMIYA camera using Kodak 120 mm film at CCT-Bahia Blanca.

3. Results

3.1. Effects of chlorpyrifos on algae growth

All assayed chlorpyrifos concentrations inhibited the growth of *A. gracilis* (Fig. 1). At the beginning of the bioassay, i.e., 24 and 48 h of exposure, treatments with 9.37, 18.75 and 37.5 mg L^{-1} of chlorpyrifos did not significantly inhibit the growth of *A. gracilis*, while higher concentrations (75 and 150 mg L^{-1}) significantly inhibited the algal growth compared with the control culture ($F=9.72$; $p=0.007$ at 24 h and $F=48.92$; $p=0.0001$ at 48 h).

As exposure time increased (i.e., 72 and 96 h time-points), statistically significant lower algae growths than in the control were observed in all treatments except at 9.37 mg L^{-1} of chlorpyrifos ($F=71.02$; $p<0.0001$ at 72 h and $F=37.84$; $p=0.0002$ at 96 h) (Table 1, Fig.1).

The cultures exposed to 150 mg L^{-1} of chlorpyrifos did not present population growth, maintaining the same cell number as that inoculated at the beginning of the bioassay.

Generation time (G_t) presented a similar response pattern to growth rate (Table 1).

The EC_{50} for the *%I* at the end of the bioassay was of 22.44 mg L^{-1} of chlorpyrifos with a confidence interval (95%CI) ranging from 18.28 to 26.80 mg L^{-1}.

3.2. Effects of chlorpyrifos on cellular size and morphology

Exposed *A. gracilis* presented an increase in cellular size in response to the increase of insecticide concentration and exposure time.

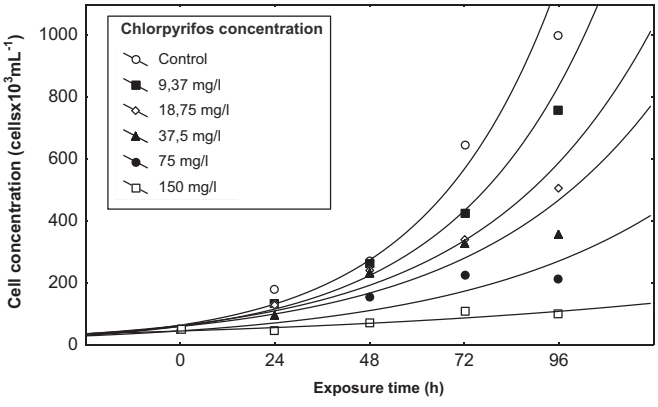

Fig. 1. Daily density of *Ankistrodesmus gracilis* (cells × 10^3 mL^{-1}) exposed to different chlorpyrifos concentrations over 96 h.

Table 1
Exponential growth rate (r) and generation time (G_t) of *Ankistrodesmus gracilis* cultures after 96 h of exposure to different concentrations of chlorpyrifos.

Chlorpyrifos concentration (mg L^{-1})	r (h^{-1})	G_t (h)
Control	0.0300	23.11
9.37	0.0270	25.68
18.75	0.0227*	30.49*
37.5	0.0191*	36.29*
75	0.0137*	50.59*
150	0.0042*	168.47*

* Significantly different from control culture (One way ANOVA; $p<0.05$).

After 48 and 72 h, cells exposed to the highest assayed concentration (150 mg L^{-1}) presented an important increase in size, resulting in cellular volumes 45.83% and 108.01% greater than measured in controls, respectively (Table 2).

At the end of the bioassay statistically significant differences were found between treatments ($H=21.38$; $p=0.0007$), cell exposed to 75 mg L^{-1} of chlorpyrifos presented statistically significant larger size than cells from the control ($p<0.05$) and from treatments with 9.37 ($p<0.05$) and 18.75 mg L^{-1} ($p<0.05$) of chlorpyrifos(Fig. 2, Table 2). At that exposure time, cells exposed to 150 mg L^{-1} of chlorpyrifos showed statistically significant differences with control culture cells and treatments with 9.37 ($p<0.05$), 18.75 ($p<0.05$) and 37.5 mg L^{-1} ($p<0.05$), biovolume was 176.25% higher than that of the control cells (Fig. 2).

3.3. Effects of chlorpyrifos on the ultrastructure of A. gracilis

At the end of the bioassay, normal cells were found only in cultures exposed to 9.37 mg L^{-1} of chlorpyrifos, while in the remaining treatments, cells with different degrees of anomalies, including chlorotic cells with abundant cytoplasmic granules were observed under the optical microscope.

Scanning electron microscopy of control culture cells showed that the cells had half-moon shape; i.e., they were slightly curved and had sharp extremes (Fig. 3A). Externally, the cell wall showed a number of irregular crests (Fig. 3B).

From the ultrastructural point of view, control culture cells showed the characteristics that best typify Chlorococcales (Dodge, 1973), including a large dictyosome located in a pronounced neckline of the nucleus (Fig. 3C). The nucleus exhibited a notorious nucleolus and condensed chromatin next to the nuclear envelope (Fig. 3C and D). The parietal chloroplasts occupied about half of the cell volume and showed two conspicuous pyrenoids and thylakoids arranged in an almost parallel pattern, and sometimes the thylakoids were separated by starch granules (Fig. 3C and D). The cytoplasm was homogeneous, showing fine and regular granulation due to the presence of ribosomes. The cell wall was characterized by the presence of three layers, the outermost of which

Table 2
Percent increase (%) in the biovolume of *Ankistrodesmus gracilis* exposed to different chlorpyrifos concentrations in relation to average cell biovolume in the control treatment.

Clorpyrifos concentration (mg L^{-1})	Exposure time (h)			
	24	48	72	96
9.37	2.39	7.62	4.08	6.37
18.75	8.84	16.72	12.61	9.12
37.5	9.91	18.33	16.96	19.76
75	18.37	29.72	30.25	77.64
150	26.77	45.83	108.01	176.25

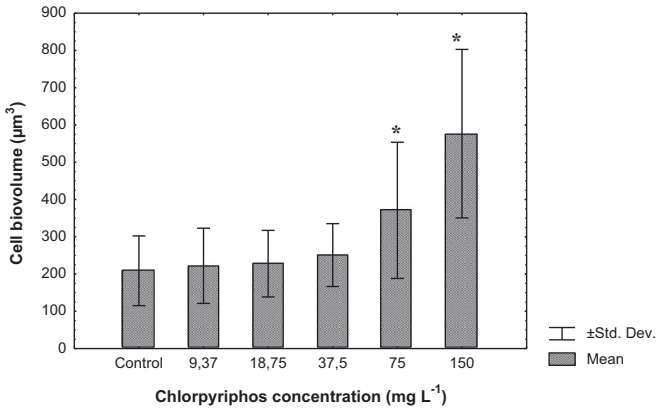

Fig. 2. Mean biovolume (μm^3) of *Ankistrodesmus gracilis* in the control and chlorpyrifos treatments after 96 h of exposure. *Statistically significant differences from the control treatment ($p < 0.05$; Kruskal–Wallis test). Std. Dev: standard deviation.

was involved in forming the crests (Fig. 3E). Autospores, which constitute the most common mode of asexual reproduction in these algae, were observed both inside the mother cells and free.

A. gracilis cells exposed to chlorpyrifos presented radical changes that increased in frequency/importance with insecticide concentration increase. By SEM, it was observed that the insecticide markedly increased cell size and widened surface crests (Fig. 4A).

Transmission electron microscopy (TEM) of cells exposed to 37.5 mg L^{-1} of chlorpyrifos showed damage in the cell wall since the three layers participated in the formation of crests, in contrast to control cells (Fig. 4B). At that insecticide concentration, electrodense bodies, presumably containing material originating from the insecticide, formed in the cytoplasm and between the thylakoids (Fig. 4B–D) as well as an increase in the number and size of starch granules (B) were observed. In the cytoplasm of some cells, the development of vacuoles was also observed (Fig. 4C).

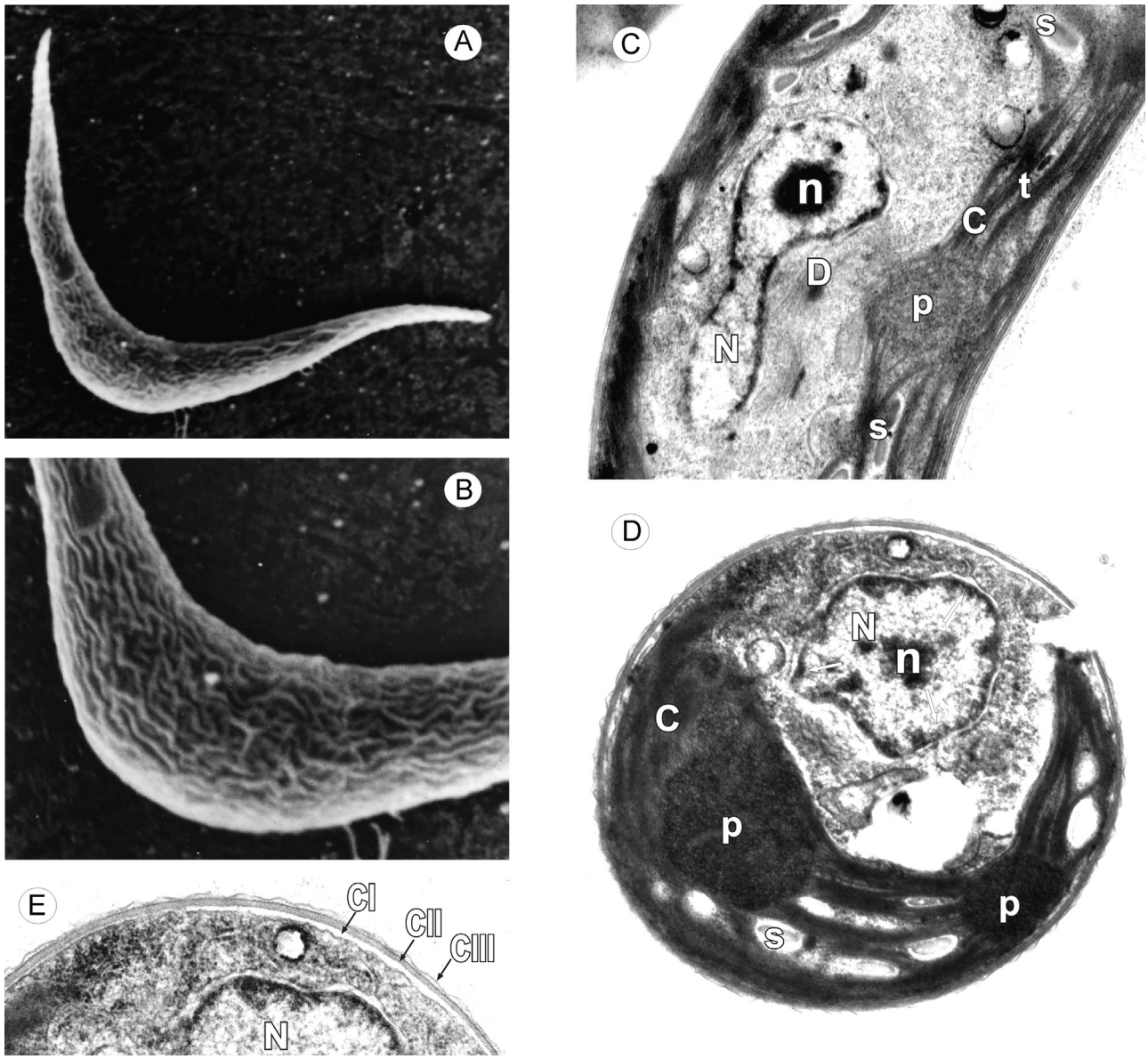

Fig. 3. *Ankistrodesmus gracilis* control treatment. (A and B) scanning electron microscope images; (A) general appearance of the cell showing the half-moon shape (× 5800); (B) detail of a cell showing the irregular crests on the cell wall (× 12,800); (C, D and E) transmission electron microscope images; (C) longitudinal section of a vegetative cell. Note the dictyosome located in a neckline of the nucleus (× 15,200); (D) cross section of a vegetative cell. Note the nucleolus and the condensed chromatin next to the nuclear envelope (white arrows) and the parietal chloroplasts occupying about half of the cell volume (× 24,500); (E) detail of the cell wall characterized by the presence of three layers (CI, CII and CIII) (× 39,000). C: chloroplast; N: nucleus; D: dictyosome; t: thylakoids; s: starch granule; n: nucleolus; p: pyrenoid.

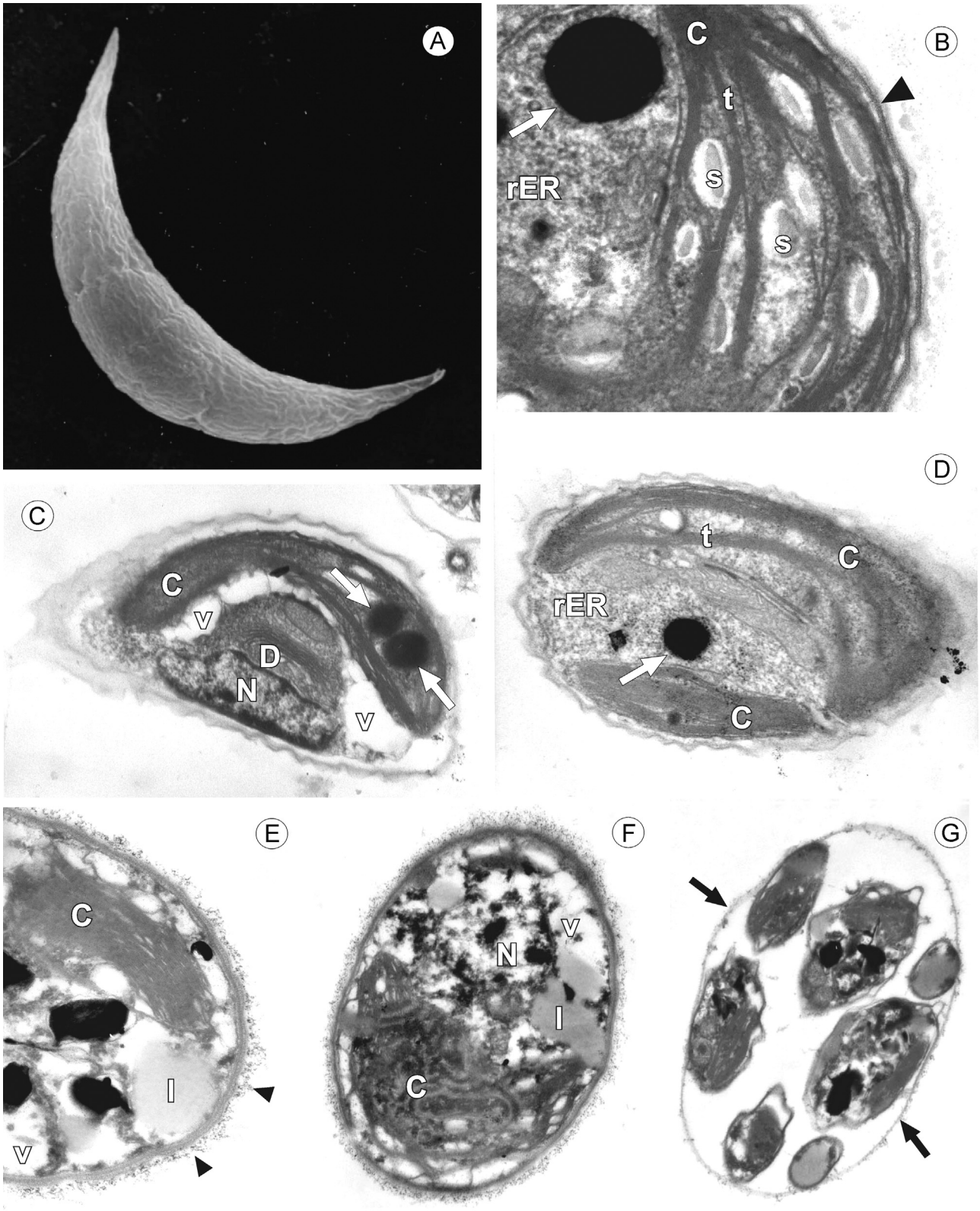

Fig. 4. *Ankistrodesmus gracilis* chlorpyrifos treatment. (A) scanning electron microscopy of vegetative cells treated with 150 mg/L of chlorpyrifos. Note the increase in the cellular size and the widening of crests (× 5700); (B, C and D) transmission electron image of vegetative cells treated with 37.5 mg L^{-1} of chlorpyrifos. Note the electrodense bodies (white arrows); (B) note that the three cell wall layers are involved in the formation of crests (arrow head) (× 40,000); (C) note the vacuoles in the cytoplasm of the cell (× 24,800); (D) note that thylakoids begin to be disorganized (× 35,500). (E, F and G) transmission electron image of vegetative cells treated with 150 mg L^{-1} of chlorpyrifos; (E) detail of a cell showing the disorganization of the cell wall and the electrodense bodies precipitated outside the wall (arrow heads) and the vacuoles and lipid globules (× 22,200); (F) cross section of a vegetative cell, note the total thylacoids disorganization (× 14,200); (G) autospores inside the mother cell (black arrows) (× 8000). C: chloroplast; N: nucleus; D: dictyosome; rER: rough endoplasmic reticulum; l: lipidic globule; v: vacuole; t: thylakoids; s: starch granule.

On the other hand, mitosis and cytokinesis were not affected with the exposure to 37.5 mg L^{-1} of chlorpyrifos and normal autospores formation was observed. Other organelles such as mitochondria, Golgi apparatus and chloroplasts were not affected by this concentration (Fig. 4B–D).

At 150 mg L^{-1} of chlorpyrifos, a disruption of all organelles in the cytoplasm was observed. There was an increase in the number of lipids and electrodense bodies which occupied almost the entire cytoplasm (Fig. 4E and F). Thylakoids were totally disrupted (Fig. 4E). The crests in the cell wall were not well defined and an electrodense precipitate was observed outside the wall (Fig. 4F). Karyokinesis occurred normally, but the cell-separation phase was inhibited at this concentration (Fig. 4G).

4. Discussion

In the present study, growth of *A. gracilis* was retarded presenting progressively decreasing growth rate upon chlorpyrifos concentration increase. In fact, concentrations equal to or higher than 18.75 mg L^{-1} of chlorpyrifos significantly inhibited the growth of *A. gracilis,* in agreement with previous studies on *S. capricornutum* (Asselborn et al., 2000). Moreover, interference with normal growth in autotrophic microorganisms has been noted with several organophosphorus insecticides including fenitrothion (Kent and Weinberger, 1991), parathion, fenthion, diazinon (Cetın et al., 2011; Lal, 1982) and pyridaphenthion (Sabater and Carrasco, 2001a).

Sabater and Carrasco (2001a) studying the effect of the organophosphorus insecticide pyridaphenthion on several freshwater species of phytoplankton found that the growth of two species of *Scenedesmus* was significantly inhibited at concentrations ranging from 0.76 to 15.6 mg L^{-1}, whereas the growth of two species of *Chlorella* was inhibited at concentrations between 11.9 and 55.2 mg L^{-1}, showing a considerable difference in sensitivity among species.

In spite of the decrease in the growth rate observed in laboratory toxicity tests, several authors have pointed out an increase in the abundance of phytoplankton in treatments with chlopyrifos in studies at population, community or ecosystem level, most commonly conducted in field or microcosm settings (Brock et al., 1992, Papst and Boyer, 1980, van Donk et al., 1995). This phenomenon is considered to be a top-down effect elicited when a predatory/grazer is more sensitive to a contaminant than its prey (Fleeger et al., 2003). Such negative impact on grazer populations leads not only to an increase in the abundance of phytoplankton, but also a change in the structure of the community.

In treatments with 75 and 150 mg L^{-1} of chlorpyrifos, a significant increase in the biovolume of *A. gracilis* cells was registered. Changes in the size of algal cells resulting from the exposure to organophosphorus insecticides have been reported by several authors. Kent and Weinberger (1991) reported an increase in the cellular volume of *S. capricornutum*, *Chlamydomonas segnis* and *Chlorella pyrenoidosa* when exposed to 1 and 10 mg L^{-1} of the organophosphorus insecticide fenitrothion. *Skeletonema costatum* giant cells were observed in treatments with the organophosphorus insecticide ethoprop (Walsh and Alexander, 1980).

With the exception of the treatment with the lowest chlorpyrifos concentration, after 96 h of exposure, cells presented different degrees of anomalies, including chlorotic cells with abundant cytoplasmic granules. The presence of chlorotic cells was also observed in bioassays with *Scenedesmus quadricauda* exposed to 50 μg L^{-1} of the organophosphorus insecticide MEP by Guanzon et al. (1996). On studying the effect of chlorpyrifos on *S. capricornutum*, Asselborn et al. (2000) also observed significant alterations in the cellular volume accompanied by depigmentation and the presence of cytoplasmic granules.

Kent and Weinberger (1991) related the increase in cellular size with the increase in the amount of macromolecules elicited by the inhibition of cell separation. Accumulation of macromolecules as a result of inhibited eukaryotic replication has been demonstrated with organochlorine (Lal and Saxena, 1980) and carbamate (DeChacin, 1984) insecticides in green algae. In the present study, the inhibition of the cell-separation phase was observed by TEM due to the exposure to 150 mg L^{-1} of chlorpyrifos, which might be responsible for the significant increase in biovolume under high concentrations of insecticide.

The increase in the cellular size may have significant ecological implications since several parameters depend on the cellular size and shape, including the growth and photosynthetic rate (Happey-Wood, 1993; Raven, 1998; Tang, 1995; Tang and Peters, 1995), the nutrient uptake (Grover, 1989; Lewis, 1976), the sinking velocity (Reynolds, 1984) and prey selection by grazers (Gliwicz, 1980). Therefore, alterations of algal cell size due to xenobiotic exposure not only implicate negative effects on higher trophic level organisms, but may also influence the competitive success of the phytoplankton.

By using electron microscopy techniques several authors have reported changes in the fine structure of cells after being exposed to different contaminants (Rachlin et al., 1985, 1982; Tukaj et al., 1998; Visviki and Rachlin, 1994; Wong et al., 1994). However, the studies about the impact of pesticides on algal ultrastructure are scarce. In the present study the most significant damage observed on the cellular ultrastructure included: formation of electrodense bodies, accumulation of lipids and increase in the size and number of starch granules.

The structure of electrodense bodies was very similar to that of polyphosphate bodies of cyanobacterias. Polyphosphate bodies not only store phosphorus and/or are an energy source in the cell, but also contain reserves of several important elements and can even accumulate toxic substances (Jensen, 1993). Compartmentalization of toxic substances, mainly heavy metals, in polyphosphate bodies has been well documented (Jensen and Rachlin, 1984; Volland et al., 2011; Wong et al., 1994); most of these authors agree that polyphosphate bodies function as a mechanism of detoxification or protection of the algae from toxicity.

The presence of large lipid bodies has also been reported by Tukaj et al. (1998) in cells of *Scenedesmus microspina* exposed to diesel fuel oil and by Rachlin et al. (1985) in cells of *Anabaena variabilis* and *A. flos-aquae* exposed to zinc. The increase in the size and number of lipid globules represents another detoxification mechanism of the cells since the lipids in algae also serve to either store or absorb pesticides in cell membranes (Guanzon et al., 1996)

An increase in the number and size of starch granules was also observed by Visviki and Rachlin (1994) in *Chlamydomonas bullosa* at 0.78 μM copper and 0.025 μM cadmium concentrations. Furthermore, Wong et al. (1994) observed an increase in the size starch granules in *Chlorella* cells exposed at 2.12 μg L^{-1} of an effluent containing high percentages of chlorophenol.

5. Conclusions

The present study demonstrates that the exposure of phytoplankton to the insecticide chlorpyrifos leads to effects observed at both cellular and population level. Growth of *A. gracilis* was inhibited after the exposure to different concentrations of chlorpyrifos with the growth rate decreasing progressively upon the increase in the pesticide concentration. In treatments with 75 and 150 mg L^{-1} of chlorpyrifos, also a significant increase in the biovolume of *A. gracilis* cells was registered. Regarding cellular

ultrastructure, the most significant damage observed included formation of electrodense bodies, accumulation of lipids and increase in the size and number of starch granules. The electrodense bodies and lipid globules would represent a detoxification mechanism since they have already been mentioned as toxic substances accumulation sites. Thus, effects of insecticides do not remain restricted to target organisms, but also extend to non-target organisms which play an important role in the ecosystem and the food chain.

Acknowledgments

Support was provided by grants to E.R.P. of the Secretaría de Ciencia y Tecnología of the Universidad Nacional del Sur, PGI CSU-B24/184. E.R.P., C.F. and Y.Z.D. are research members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

References

Anton, F.A., Ariz, M., Alia, M., 1993. Ecotoxic effects of four herbicides (glyphosate, alachlor, chlortoluron and isoproturon) on the algae *Chlorella pyrenoidosa* Chick. Sci. Total Environ. 134, 845–851.

Asselborn, V., Zalocar de Domitrovic, Y., Parodi, E.R., 2000. Efectos del insecticida organofosforado chorpyriphos sobre el crecimiento y morfología de *Selenastrum capricornutum* Printz (Chlorophyta). In: Espíndola, E.L.G., Botta-Paschoal, C.M.R., Rocha, O., Boherer, M.B.C., Lopes de Oliveira Neto, A. (Eds.), Ecotoxicologia Perspectivas para o Século XXI. RiMa, São Carlos, São Paulo, pp. 281–291.

Atici, T., Ahiska, S., Altinda, A., Aydin, D., 2008. Ecological effects of some heavy metals (Cd, Pb, Hg, Cr) pollution of phytoplanktonic algae and zooplanktonic organisms in Sarıyar Dam Reservoir in Turkey. Afr. J. Biotechnol. 7, 1972–1977.

Boltovskoy, A., 1995. Taxonomía y morfología de los dinoflagelados: Métodos de trabajo. In: Alveal, K., Ferrario, M.E., Oliveira, E.C., Sar, E. (Eds.), Manual de métodos ficológicos. Universidad de Concepción, Concepción, pp. 55–82.

Brock, T.C.M., van den Bogaert, M., Bos, A.R., van Breukelen, S.W.F., Reiche, R., Terwoert, J., Suykerbuyk, R.E.M., Roijackers, R.M.M., 1992. Fate and effects of the insecticide Dursban 4e in indoor *elodea*-dominated and macrophyte-free freshwater model ecosystems: II. Secondary effects on community structure. Arch. Environ. Contam. Toxicol. 23, 391–409.

Cáceres, E., 1995. Métodos de preparación de algas para su observación con microscopía electrónica de transmisión convencional (METC). In: Alveal, K., Ferrario, M.E., Oliveira, E.C., Sar, E. (Eds.), Manual de métodos ficológicos. Universidad de Concepción, Concepción, pp. 147–168.

Carvalho, F.P., Gonzalez-Farias, F., Villeneuve, J.-P., Cattini, C., Hernandez-Garza, M., Mee, L.D., Fowler, S.W., 2002. Distribution, fate and effects of pesticide residues in tropical coastal lagoons of northwestern Mexico. Environ. Technol. 23, 1257–1270.

CASAFE (Cámara de sanidad agropecuaria y fertilizantes), 2009. Guía de productos fitosanitarios para la República Argentina. Buenos Aires, Argentina.

Cetın, A.K., Gur, N., Fırat, Z., 2011. Growth rate of *Scenedesmus acutus* in laboratory cultures exposed to diazinon. Afr. J. Biotechnol. 10, 6540–6543.

DeChacin, C., 1984. The effects of aminocarb and its adjuvants in the Matacil 1.8D formulation on the cell cycle and macromolecular content of *Chlamydomonas segnis*, ETTL. (Dissertation). University of Ottawa, Canada.

Dodge, J.D., 1973. The Fine Structure of Algal Cells. Academic Press, London, New York.

Domitrovic, H.A., 1997. Histología e histopatología de Aequidens portalegrensis (Hensel 1870): 1. Morfología normal y patologías normales. 2. Ensayos con agentes terapéuticos y pesticidas (Dissertation). University of Buenos Aires, Argentina.

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, London.

Fleeger, J.W., Carman, K.R., Nisbet, R.M., 2003. Indirect effects of contaminants in aquatic ecosystems. Sci. Total Environ. 317, 207–233.

Fogg, G.E., 1975. Algal Cultures and Phytoplankton Ecology. The University of Wisconsin Press, USA.

Gliwicz, Z.M., 1980. Filtering rates, food size selection and feeding rates in cladocerans: another aspect of interspecific competition in filter-feeding zooplankton. In: Kerfoot, W.C. (Ed.), Evolution and Ecology of Zooplankton Communities. University of New England Press, Hanover, pp. 282–291.

Gómez de Barreda Ferraz, D., Sabater, C., Carrasco, J.M., 2004. Effects of propanil, tebufenozide and mefenacet on growth of four freshwater species of phytoplankton: a microplate bioassay. Chemosphere 56, 315–320.

Grover, J.P., 1989. Influence of cell shape and size on algal competitive ability. J. Phycol. 25, 402–405.

Guanzon, N.G., Fukuda, M., Nakahara, H., 1996. Accumulation of agricultural pesticides by three freshwater microalgae. Fish. Sci. 62, 690–697.

Happey-Wood, C.M., 1993. Diurnal and seasonal variation in the contributions of autotrophic pico-, nano- and microplankton to the primary production of an upland lake. J. Plankton Res. 15, 125–159.

Jensen, T.E., 1993. Cyanobacterial ultrastructure. In: Berner, T. (Ed.), Ultrastructure of Microalgae. CRC Press, Florida, pp. 7–51.

Jensen, T.E., Rachlin, J.W., 1984. Effect of varying sulphur deficiency on structural components of a cyanobacterium *Synechococcus leopoliensis*: a morphometric study. Cytobios 41, 35–46.

Katsumata, M., Koikke, T., Nishikawa, M., Kazumura, K., Tsuchiya, H., 2006. Rapid ecotoxicological bioassay using delayed fluorescence in the green algal *Pseudokircneriella subcapitata*. Water Res. 40, 3393–3400.

Kent, R.A., Weinberger, P., 1991. Multibiological-level responses of freshwater phytoplankton to pesticides stress. Environ. Toxicol. Chem. 10, 209–216.

Lacorte, S., Lartiges, S.B., Garrigues, P., Barcelo, D., 1995. Degradation of organophosphoms pesticides and their transformation products in estuarine waters. Environ. Sci. Technol. 29, 431–438.

Lal, R., 1982. Accumulation, metabolism and effect of organophosphorus insecticides on microorganisms. Adv. Appl. Microbiol. 28, 149–200.

Lal, R., Saxena, D.M., 1980. Cytological and biochemical effects of pesticides on microorganisms. Residue Rev. 73, 49–86.

Larson, S.J., Capel, P.D., Majewski, M.S., 1997. Pesticides in Surface Waters: Distributions, Trends, and Governing Factors. Ann Arbor Press, Chelsea, MI, USA.

Lewis, W.M., 1976. Surface/volume ratio: implications for phytoplankton morphology. Science 192, 885–887.

Martinez, R.S., Di Marzio, W.D., Saénz, M.E., 2015. Genotoxic effects of commercial formulations of Chlorpyrifos and Tebuconazole on green algae. Ecotoxicology 24, 45–54.

Mayer-Pinto, M., Coleman, R.A., Underwood, A.J., Tolhurst, T.J., 2011. Effects of zinc on microalgal biofilms in intertidal and subtidal habitats. Biofouling 27, 721–727.

Moro, C.V., Bricheux, G., Portelli, C., Bohatier, J., 2012. Comparative effects of the herbicides chlortoluron and mesotrione on freshwater microalgae. Environ. Toxicol. Chem. 31, 778–786.

OECD (Organisation for Economic Co-operation and Development), 2006. OECD Guidelines for the Testing of Chemicals. Section 2: Effects on Biotic Systems. Test 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Paris.

Papst, M.H., Boyer, M.G., 1980. Effects of two organophosphorus insecticides on the chlorophyll a and pheopigment concentrations of standing ponds. Hydrobiologia 69, 245–250.

Rachlin, J.W., Jensen, T.E., Baxter, M., Jani, V., 1982. Utilization of morphometric analysis in evaluating response of *Plectonema boryanum* (Cyanophyceae) to exposure to eight heavy metals. Arch. Environ. Contam. Toxicol. 11, 323–333.

Rachlin, J.W., Jensen, T.E., Warkentine, B.E., 1985. Morphometric analysis of the response of *Anabaena flos-aquae* and *Anabaena variabilis* (Cyanophyceae) to selected concentrations of zinc. Arch. Environ. Contam. Toxicol. 14, 395–402.

Raven, J.A., 1998. Small is beautiful: the picophytoplankton. Funct. Ecol. 12, 503–513.

Reymond, O.L., Pickett-Heaps, J.D., 1982. A routine flat embedding method for electron microscopy of microorganisms allowing selection and precisely oriented sectioning of single cells by light microscopy. J. Microsc. 130, 79–84.

Reynolds, C.S., 1984. The Ecology of Freshwater Phytoplankton. Cambridge University Press, Cambridge.

Rioboo, C., González, O., Herrero, C., Cid, A., 2002. Physiological response of freshwater microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides. Aquat. Toxicol. 59, 225–235.

Sabater, C., Carrasco, J.M., 2001a. Effects of pyridaphenthion on growth of five freshwater species of phytoplankton. A laboratory study. Chemosphere 44, 1775–1781.

Sabater, C., Carrasco, J.M., 2001b. Effects of the organophosphorus insecticide fenitrothion on growth in five freshwater species of phytoplankton. Environ. Toxicol. 16, 314–320.

Sabater, C., Cuesta, A., Carrasco, R., 2002. Effects of bensulfuron-methyl and cinosulfuron on growth of four freshwater species of phytoplankton. Chemosphere 46, 953–960.

Sáenz, M.E., Accorinti, J., Tortorelli, M., del, C., 1993. Toxicity of paraquat to a green algae *Scenedesmus acutus*. J. Environ. Sci. Health B 28, 193–204.

Sáenz, M.E., Di Marzio, W.D., Alberdi, J.L., Tortorelli, M., del, C., 1997. Effects of technical grade and a commercial formulation of gliphosate on algal population growth. Bull. Environ. Contam. Toxicol. 59, 638–644.

Schiff, K., Bay, S., Stransky, C., 2002. Characterization of storm water toxicants from an urban watershed to freshwater and marine organisms. Urban Water 4, 215–227.

Solomon, K.R., Williams, W.M., Mackay, D., Purdy, J., Giddings, J.M., Giesy, J.P., 2014. Properties and uses of chlorpyrifos in the United States. In: Giesy. J.P., Solomon, K.R. (Eds.) Ecological risk assessment for chlorpyrifos in terrestrial and aquatic systems in the United States. Reviews of Environmental Contamination and Toxicology, 231, 13–34.

Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31–43.

Stein, J.R., 1973. Handbook of Phycological Methods. Culture Methods and Growth Measurements.. Cambridge University Press, London.

Stratton, G.W., 1987. The effects of pesticides and heavy metals towards phototrophic microorganisms. Rev. Environ. Toxicol. 3, 71–147.

Streit, B., Kuhn, K., 1994. Effects of organophosphorous insecticides on autochthonous and introduced Gammarus species. Water Sci. Technol. 29, 233–240.

Sun, J., Liu, D., 2003. Geometric models for calculating cell biovolume and surface

area for phytoplankton. J. Plankton Res. 25, 1331–1346.
Tang, E.P.Y., 1995. The allometry of algal growth rates. J. Plankton Res. 17, 1325–1335.
Tang, E.P.Y., Peters, R.H., 1995. The allometry of algal respiration. J. Plankton Res. 17, 303–315.
Tukaj, Z., Bohdanowicz, J., Aksmann, A., 1998. A morphometric and stereological analysis of ultrastructural changes in two *Scenedesmus* (Chlorococcales, Chlorophyta) strains subjected to diesel fuel oil pollution. Phycologia 37, 388–393.
USEPA (U.S. Environmental Protection Agency), 1986. Ambient Water Quality Criteria for Chlorpyrifos. Washington, DC. EPA 440/5-86-005.
USEPA (U.S. Environmental Protection Agency), 1989. In short-term methods for estimating the chronic toxicity of effluents and receiving water to freshwater organisms. Environmental Monitoring and Support Laboratory Office of Research and Development, Cincinnati, Ohio.
van Donk, E., Prins, H., Voogd, H.M., Crum, S.J.H., Brock, T.C.M., 1995. Effects of nutrient loading and insecticide application on the ecology of *Elodea*-dominated freshwater microcosms I. Responses of plankton and zooplanktivorous insects. Arch. Hydrobiol. 133, 417–439.
Varó, I., Serrano, R., Navarro, J.C., Lopez, F.J., Amat, F., 1998. Acute lethal toxicity of the organophosphorus pesticide chlorpyrifos to different species and strains of *Artemia*. Bull. Environ. Contam. Toxicol. 61, 778–785.
Vendrell, E., Ferraz, D.G., Sabater, C., Carrasco, J.M., 2009. Effect of glyphosate on growth of four freshwater species of phytoplankton: a microplate bioassay. Bull. Environ. Contam. Toxicol. 82, 538–542.
Visviki, I., Rachlin, J.W., 1994. Acute and chronic exposure of *Dunaliella salina* and *Chlamydomonas bullosa* to copper and cadmium: effects on ultrastructure. Arch. Environ. Contam. Toxicol. 26, 154–162.
Volland, S., Andosch, A., Milla, M., Stöger, B., Lütz, C., Lütz-Meindl, U., 2011. Intracellular metal compartmentalization in the green algal model system *Micrasterias denticulata* (Streptophyta) measured by transmission electron microscopy-coupled electron energy loss spectroscopy. J. Phycol. 47, 565–579.
Walsh, G., Alexander, S.V., 1980. A marine algal bioassay method results with pesticides and industrial wastes. Water Air Soil Pollut. 13, 45–55.
Wang, X., Wang, W.X., 2005. Uptake, absorption efficiency and elimination of DDT in marine phytoplankton, copepods and fish. Environ. Pollut. 136, 453–464.
Wendt-Rasch, L., Pirzadeh, P., Woin, P., 2003. Effects of metsulfuron methyl and cypermethrin exposure on freshwater model ecosystems. Aquat. Toxicol. 63, 243–256.
Wong, P.K., Chang, L., 1988. The effects and organophosphorus insecticides on growth, photosynthesis, and chlorophyll *a* synthesis of *Chlamydomonas reinhardtii* (mt+). Environ. Pollut. 55, 179–189.
Wong, S.L., Nakamoto, L., Wainwright, J.F., 1994. Identification of toxic metals in affected algal cells in assays of wastewaters. J. Appl. Phycol. 6, 405–414.
Zar, J.H., 2009. Biostatistical Analysis. Prentice Hall, Upper Saddle River, NJ.