Physiological response of freshwater microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides

C Rioboo, O González, C Herrero, A Cid,[1]

Aquatic Toxicology, Volume 59, Issues 3–4, 24 September 2002, Pages 225–235

Received 4 May 2001, Revised 20 July 2001, Accepted 18 October 2001, Available online 27 November 2001

DOI: 10.1016/S0166-445X(01)00255-7

Abstract

The effects of two herbicides used wide-spread, isoproturon (phenylurea) and terbutryn (triazine), on growth, dry weight, elemental composition, photosynthetic pigments and protein content, and cell volume assayed by flow cytometry in the freshwater microalgae *Chlorella vulgaris* were studied. Different parameters for algal activity show widely different sensitivities to these aquatic pollutants. After 96 h of herbicide exposure, terbutryn was the strongest inhibitor of growth, giving an EC_{50} value for growth twice lower than that for isoproturon cultures (EC_{50} terbutryn=0.097 μM; EC_{50} isoproturon=0.199 μM). However, lower concentrations of the triazine herbicide provoked an increase in the cellular density and growth rate of this microalga, not observed in the phenylurea-treated cultures. Cellular volume and dry weight of *C. vulgaris* cells were increased strongly in the presence of isoproturon and terbutryn. Other cellular parameters, such as pigment and protein content, were stimulated with both herbicides at higher concentrations.

Keywords

Microalga; Herbicide; Triazine; Phenylurea; Toxic effects

1. Introduction

Most phytotoxicological research with herbicides has been conducted on target plants (i.e. efficacy studies on weeds). Little data exist on the effects of these pollutants may have in aquatic systems (Caux et al., 1996), excepting the triazine atrazine. The sensitivity of algae to many herbicides is very high, and a better understanding of their environmental effects is

[1] Laboratorio de Microbioloxía, Facultad de Ciencias, Universidade da Coruña, Rúa Alejandro de la Sota no. 1, 15008 A Coruña, Spain

probably acquired by using test species representing non-target groups (Haglund, 1997). As the major primary producers in freshwater ecosystems, microalgae play a pivotal role in the functioning of a healthy ecosystem. Because of their short generation times, microalgae respond rapidly to environmental changes and, thus, may report impacts on higher organisms, which generally respond on longer time scales (McCormick and Cairns, 1994). 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). For example, algal toxicity tests of chemicals are mandatory tests for notification of chemicals in the European Union countries (Organization for Economic Cooperation and Development (OECD), Algae growth inhibition test, Test Guidelines, OECD Guideline for Testing of Chemicals, Paris, no. 201, p. 14, 1984). Other fields of use for microalgae in toxicity assessment are industrial wastewaters and leachates from waste deposits (Sosak-Swiderska et al., 1998).

Isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea) is a pre- or post-emergence systemic herbicide, commonly used to control annual grasses and broad-leaved weeds in barley, wheat and rye, relatively soluble in water (Tomlin, 1994). Very little research has been devoted to the study of isoproturon and its toxic effects on primary producers in freshwater systems (Pérés et al., 1996).

Terbutryn (*N*^{2}-*tert*-butyl-*N*^{4}-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) is also a pre- or post-emergence systemic herbicide. It is used to control most grasses and many annual broad-leaved weeds in winter cereals, potatoes, legumes, sunflowers, maize, sugar cane and citrus fruit. Furthermore, it is used as an aquatic herbicide for controlling submerged and free-floating weeds and algae in water courses (Tomlin, 1994).

Photosynthesis is a primary and effective target site for over 50% of the commercially available herbicides (Singh et al., 1997). Isoproturon and terbutryn have been used as herbicides in large amounts since the mid-1980s (Larsen et al., 2000).

These herbicides of urea (isoproturon) and triazine (terbutryn) classes act biochemically by displacing a plastoquinone (Q_B) from its binding site in the D1 protein of photosystem II (PS II), i.e. their primary site of action being inhibition of the Hill reaction of photosynthetic electron transport (Cremlyn, 1991).

In this study, herbicide

phytotoxicity assessment was conducted in the laboratory on the freshwater green algae (*Chlorella vulgaris*), one of the most commonly used species in microalgal toxicity tests (Nalewajko and Olaveson, 1998). Parameters, such as growth and photosynthetic pigment content, frequently used in microalgal toxicity assays were examined. Furthermore, cell volume analysed by flow cytometry was used to compare with cell dry weight data.

2. Materials and methods

2.1. Microalgal cultures

Chlorella vulgaris Beijerinck (Oocystaceae) was obtained from the Culture Collection of Algae and Protozoa of the Institute of Freshwater Ecology (Cumbria, UK) (strain CCAP 211/11B) and was maintained in a Bristol medium (Brown et al., 1967). The inoculum was taken from a 3-day-old culture, with the aim to use cells growing in a logarithmic phase for all experiments. Cultures were grown in autoclaved (121 °C, 20 min) 500-ml Pyrex glass bottles containing 300 ml of medium. Microalgal cultures were maintained at 18±1 °C and 68.25 μmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12 h. Initial cell density for each experiment was 9×10^6cells ml^{-1}.

Terbutryn and isoproturon were Riedel-de Häen Pestanal (RdH Laborchemikalien GmbH & Co.; Seelze, Germany) standards for environmental analysis. Herbicide stock solutions were prepared by dissolving granulated herbicides in 100% methanol. Terbutryn concentrations assayed were 0.025, 0.05, 0.10 and 0.20 μM, while isoproturon concentrations assayed were 0.05, 0.10, 0.25 and 0.50 μM. Final methanol concentrations for each treatment did not exceed 0.05% (v/v), and no measurable effects on growth or on other parameters assayed were observed when this concentration of methanol was tested in the absence of herbicides. Cultures without herbicide were included as control in all experiments. All experiments were carried out in triplicate for 96 h as has already been indicated for toxicity assays with microalgae (Walsh and Merril, 1984).

2.2. Measurement of growth

Growth of the microalgal cultures was measured by counting daily culture aliquots in a Neubauer haemocytometer using an Eclipse E400 microscope (Nikon, Japan), and growth rates (μ), expressed in day^{-1}, were calculated by the usual formula:

$$\mu = [(\ln N_t - \ln N_0)] / \ln 2(t - t_0)$$

where N is the cell density at time t (expressed in days) after herbicide exposure.

The most common parameter used in toxicity assays is the EC_{50}, i.e. the concentration of the tested substance that decreases the growth rate by 50%. EC_{50} values after 48 and 96 h of herbicide exposure were obtained by graphic interpolation from concentration–response curves. Growth rate data analysis was carried out using the Regression Wizard (SigmaPlot 4.0, SPSS Inc.) software. Data were fitted by a non-linear regression three parameters sigmoidal curve, using Marquardt's algorithm which theoretical mathematical function is:

$$y = a / [1 + \exp(-(x - x_0) / b)]$$

where y is the percentage of the growth rate variation respect to the control (cultures without pesticide); $x$is the pesticide concentration (expressed as μM); the three parameters for this function determine the high value (a), the width of the transition (b), and the x value at which the function is 50% of the functions amplitude (x_0, closely related to EC_{50}).

2.3. Determination of cell volume

Microalgal cellular volume variations were determined after 96 h of herbicides exposure by flow cytometry, since an increase in the forward light scatter (FSC) signal can be correlated with an increase in cell volume (Shapiro, 1995). Aliquots of microalgal cultures were analyzed in a FACScan flow cytometer (Becton Dickinson Instruments, San José, CA), equipped with an argon-ion excitation laser (blue light, 488 nm). At least 10^4 cells were analysed per culture. Data collection was performed using the list-mode. The mean of forward scatter signal distributions was provided by the instrument software (LYSIS II program; Becton Dickinson Instruments, San José, CA). Data on the forward scatter of the cells, related to cell volume, were expressed as a percentage of the control cells signal (arbitrary units) according to the equation of Reader et al. (1993):

$$\%V = 100 - [100(V_c - V_t) / V_c]$$

where $\%V$ is the percentage of the FSC signal of *C. vulgaris* cells; V_c is the mean FSC signal of control cells; and V_t is the mean FSC signal of herbicide-treated cells.

2.4. Dry weight, carbon/nitrogen ratio and protein content

Samples for these analysis were taken after 96 h of herbicide exposure. Microalgal cells were harvested by centrifugation at 3000×*g* and frozen at −70 °C prior to lyophilisation for 24 h. The dry weight was determined according to Utting (1985). Carbon and nitrogen content were obtained with an elemental analyser (Carlo Erba CHNS-O 1108).

Proteins were determined from the data of nitrogen content, using the conversion factor, 5.8, described byGnaiger and Bitterlich (1984), instead of the classical conversion factor, 6.25 (FAO/WHO, 1973), since it was demonstrated that this new conversion factor is better for different kind of samples, including algae, bacteria, protozoan and invertebrates (Gnaiger and Bitterlich, 1984).

2.5. Photosynthetic pigments content

C. vulgaris photosynthetic pigment contents were determined from spectrophotometric measurements in a Shimadzu UV-160 (Japan) of 100% methanol extracts using the equations described in Lichtenthaler (1987).

Data were statistically analysed by an overall one-way analysis of variance (ANOVA) and, when differences observed were significant, the means were compared by multiple range Duncan test, at a level of significance of 0.05 (P<0.05).

3. Results

3.1. Isoproturon

3.1.1. Growth

This herbicide affected the growth of the freshwater microalga *C. vulgaris* (Fig. 1; Table 1). After 48 h, all microalgal cultures exposed to isoproturon showed a decrease in the growth rate with respect to the control cultures ($P<0.05$) (Table 1). However, no significant differences were observed between control cultures and cultures with the minimum concentration assayed, 0.05 μM ($P<0.05$) after 96 h; growth rates were 0.82 and 0.75 day^{-1}, respectively. Growth decreased as isoproturon concentration increased in the cultures; 0.50 μM drastically inhibiting microalgal growth, growth rate being below 0 (−14 day exp 1) after 96 h (Table 1).

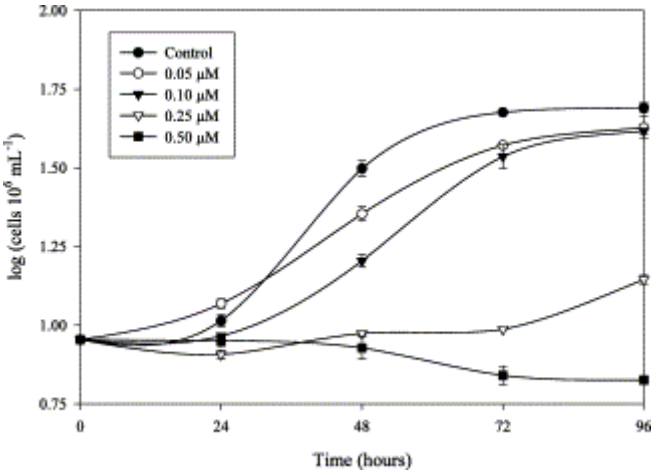

Fig. 1.

Growth curves of cultures of *C. vulgaris* exposed to different isoproturon concentrations (μM). Data are given as mean values±S.D. of the means.

Table 1.

Growth rate (GR) after 48 and 96 h, and dry weight, cell volume, protein content, and carbon/nitrogen ratio obtained in cultures of *C. vulgaris* exposed to different isoproturon concentrations after 96 h

Isoproturon (μM)	48 h GR (μ) (day^{-1})	96 h GR (μ) (day^{-1})	Dry weight (pg $cell^{-1}$)	Cell volume (%FSC)	Protein content (pg $cell^{-1}$)	C/N
0	0.90±0.03	0.82±0.01	4.48±0.09	100±0.08	2.27±0.09	5.54±0.06
0.05	0.66±0.02	0.75±0.03	5.71±0.77	141±6.68	2.99±0.04	5.43±0.06
0.10	0.42±0.02	0.73±0.02	4.27±0.15	164±6.70	2.66±0.07	5.36±0.09
0.25	0.04±0.01	0.21±0.01	10.23±0.31	270±5.45	5.59±0.11	5.21±0.12
0.50	−0.04±0.01	−0.14±0.02	14.47±1.00	294±5.80	8.59±0.14	4.59±0.07

Data are given as mean values±S.D. of the means.

Microalgal growth is characterized by a sigmoid or logistic function(Schanz and Zahler, 1981). In the present study, the growth of the microalgal cultures was fitted to the parameters of a logistic function, except for the 0.25 and 0.50 μM cultures (Fig. 1). *C. vulgaris* cultures showed a prolonged lag phase after exposure to isoproturon, reaching the stationary phase later than control.

The concentration–response curves and their mathematical functions are shown in Fig. 2. In the isoproturon cultures, the EC_{50} value after 96 h of herbicide exposure was twice the EC_{50} for the cultures exposed 48 h to isoproturon (Fig. 2).

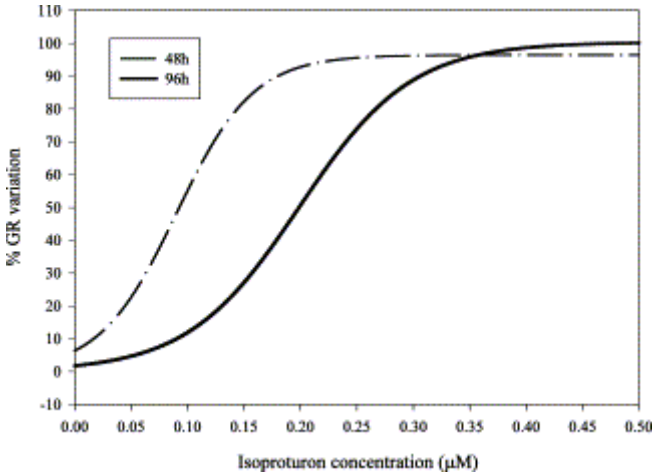

Fig. 2.

Concentration–response curves represented by growth rate (GR) variation respect to the control of cultures of *C. vulgaris*vs. different isoproturon concentrations (µM) after 48 and 96 h of herbicide exposure. 48 h: y=96.434/(1+exp(−(x−0.090)/0.034) (r=0.99) EC_{50}=0.092; 96 h: y=100.321/(1+exp(−(x−0.199)/0.049) (r=0.99) EC_{50}=0.199.

3.1.2. Cell volume

High isoproturon concentrations caused an increase in the FSC signal, related to an increase in the cell volume of *C. vulgaris*. After 96 h of herbicide exposure, the most drastic increase in cell volume was detected at isoproturon concentrations of 0.25 and 0.50 µM (270 and 294%, respectively) relative to control (P<0.05) (100%) (Table 1).

3.1.3. Dry weight, carbon/nitrogen ratio and protein content

Concentrations of 0.25 and 0.50 µM lead to a significant increase in cellular dry weight (DW) after 96 h (P<0.05), being twice to three times greater than the DW of the control culture cells (4.48 pg $cell^{-1}$) (Table 1).

Carbon and nitrogen percentages in dry biomass, determined after 96 h of culture, showed that the C/N ratio decreased significantly (P<0.05) in cultures with 0.25 and 0.50 µM (5.21 and 4.59, respectively) in comparison with the ratio obtained in control cultures (5.54) (Table 1).

Cellular protein content after 96 h of isoproturon exposure showed a significant increase (P<0.05) when the herbicide concentrations were higher than 0.10 µM, with a maximum value of 8.59 pg $cell^{-1}$ in cultures exposed to an isoproturon concentration of 0.50 µM, being nearly four times higher than the protein content of the control cells (2.27 pg $cell^{-1}$) (Table 1).

3.1.4. Photosynthetic pigments content

After 96 h of isoproturon exposure, cellular chlorophyll *a* and *b* contents were more affected than total carotenoid content by the addition of the herbicide (Fig. 3). Maximum chlorophylls content was obtained in 0.25 µM isoproturon cultures, with values of 0.38 and 0.14 pg $cell^{-1}$ of chlorophyll *a* content and chlorophyll *b*content, respectively, in comparison with the values obtained in control cultures (0.14 and 0.05 pg $cell^{-1}$, respectively) (Fig. 3). However, cells

exposed to the highest isoproturon concentration assayed showed a decrease in these chlorophylls with respect to the 0.25 μM cultures. Total carotenoid content was also increased, to 0.08 pg cell^{-1} at the highest isoproturon concentration assayed (P<0.05), while lower concentrations did not cause significant changes with respect to control cells (0.03 pg cell^{-1}) (Fig. 3).

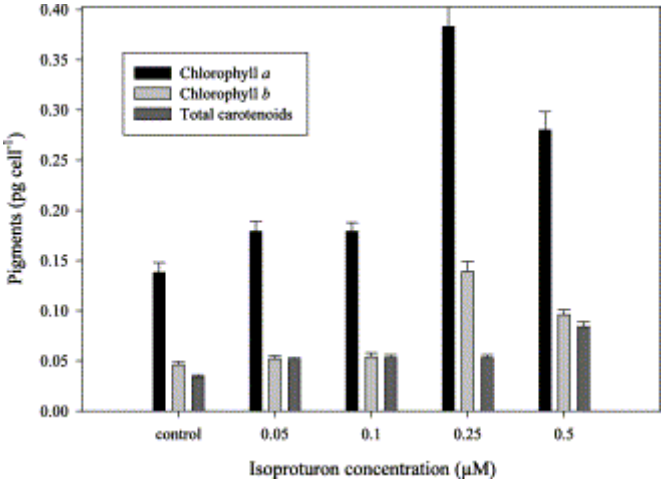

Fig. 3.

Photosynthetic pigments (chlorophylls *a* and *b*, and total carotenoids), obtained from cultures of *C. vulgaris* exposed to different isoproturon concentrations (μM) after 96 h. Data are given as mean values ±S.D. of the means.

3.2. Terbutryn

3.2.1. Growth

Growth of *C. vulgaris* cultures was affected by the addition of terbutryn to the medium (Fig. 4; Table 2). After 48 h of herbicide exposure, the most important decrease in growth rate respect to control cultures was detected at terbutryn concentrations of 0.10 and 0.20 μM (−0.06 and −0.02 days^{-1}, respectively) (P<0.05). After 96 h of herbicide exposure, higher terbutryn concentrations brought about an important decrease in growth rate, being close to zero with the maximum concentration assayed (Table 2); however, the lowest concentration assayed provoked a significant increase of growth respect to control cultures (0.92 and 0.82 days^{-1}, respectively), which are not significant different to those cultures exposed to 0.05 μM terbutryn.

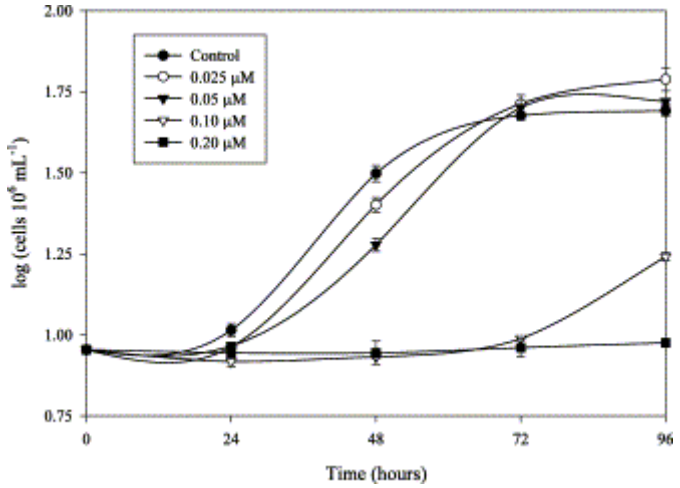

Fig. 4.

Growth curves of cultures of *C. vulgaris* exposed to different terbutryn concentrations (µM). Data are given as mean values±S.D. of the means.

Table 2.

Growth rate (GR) after 48 and 96 h, and dry weight, cell volume, protein content, and carbon/nitrogen ratio obtained in cultures of *C. vulgaris* exposed to different terbutryn concentrations after 96 h

Terbutryn (µM)	48 h GR (μ) (day^{-1})	96 h GR (µ) (day^{-1})	Dry weight (pg $cell^{-1}$)	Cell volume (% FSC)	Protein content (pg $cell^{-1}$)	C/N
0	0.90±0.03	0.82±0.01	4.48±0.09	100±0.08	2.27±0.09	5.54±0.06
0.025	0.74±0.03	0.92±0.03	4.33±0.33	117±5.34	2.25±0.10	5.38±0.05
0.05	0.54±0.02	0.85±0.01	5.24±0.16	124±12.40	2.77±0.05	5.31±0.1
0.10	−0.06±0.03	0.32±0.01	8.60±0.19	153±11.30	4.61±0.08	5.15±0.19
0.20	−0.02±0.01	0.02±0.00	6.35±0.40	147±10.10	3.46±0.12	4.93±0.09

Data are given as mean values±standard desviation of the means.

Microalgal growth is characterized by a sigmoid or logistic function (Schanz and Zahler, 1981). Growth curves of the microalgal cultures exposed to the lowest terbutryn concentrations assayed (0.025 and 0.05 µM) were fitted to the parameters of a logistic function (Fig. 4). *C. vulgaris* cultures showed a prolonged lag phase after exposure to terbutryn, reaching the stationary phase later than control.

The concentration–response curves and their mathematical functions are shown in Fig. 5 In the terbutryn cultures, the EC_{50} value after 96 h of herbicide exposure was twice the EC_{50} for the cultures exposed 48 h to terbutryn (Fig. 5).

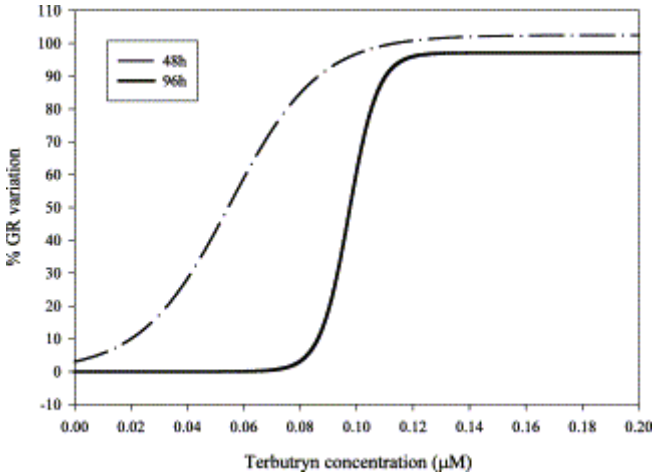

Fig. 5.

Concentration–response curves represented by growth rate (GR) variation respect to the control of cultures of *C. vulgaris* versus different terbutryn concentrations (µM) after 48 and 96 h of herbicide exposure. 48 h: y=102.485/(1+exp(−(x−0.055)/0.016) (r=0.99) EC_{50}=0.055; 96 h: y=97.101/(1+exp(−(x−0.097)/0.005) (r=0.99) EC_{50}=0.097.

3.2.2. Cell volume

This herbicide also caused an increase in the FSC signal after 96 h of exposure. The highest concentrations tested (0.10 and 0.20 µM) caused the maximum increases (P<0.05) of this signal (153 and 147%, respectively) with respect to the control (100%) (Table 2).

3.2.3. Dry weight, carbon/nitrogen ratio and protein content

Concentrations of 0.05 µM or higher provoked a significant increase (P<0.05) in cellular dry weight, giving values in the range 5.25–8.60 pg $cell^{-1}$, in comparison with the value obtained in control culture cells (4.48 pg $cell^{-1}$) (Table 2).

The C/N ratio also decreased significantly (P<0.05) in cultures exposed to the highest terbutryn concentrations assayed, 0.10 and 0.20 µM (5.15 and 4.96, respectively) relative to control cells (5.54) (Table 2).

Terbutryn concentrations of 0.05 µM or higher produced a significant increase (P<0.05) in the protein content of the cells, after 96 h of exposure, being maximum at 0.10 µM with a protein content of 4.61 pg $cell^{-1}$, twice that of control (2.27 pg $cell^{-1}$) (Table 2).

3.2.4. Photosynthetic pigments content

After 96 h of treatment, the two highest terbutryn concentrations (0.10 and 0.20 µM) brought about a significant increase both in chlorophylls and carotenoid contents of *C. vulgaris* cells with respect to the control cultures (Fig. 6).

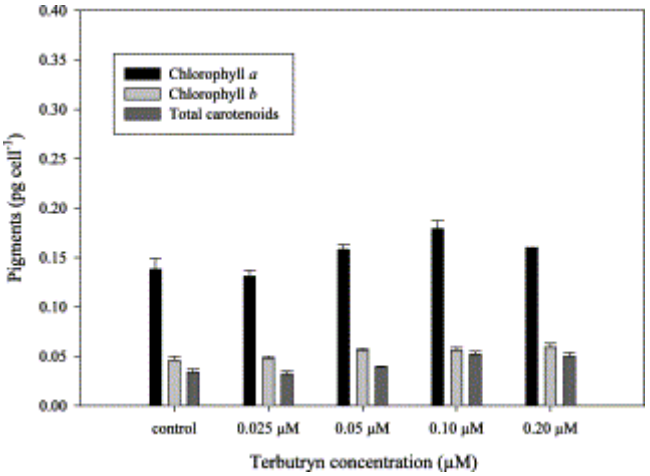

Fig. 6.

Photosynthetic pigments (chlorophylls *a* and *b*, and total carotenoids), obtained from cultures of *C. vulgaris* exposed to different terbutryn concentrations (µM) after 96 h. Data are given as mean values± S.D. of the means.

4. Discussion

Despite the fact that the effects of both herbicides might be similar, both of them are photosynthetic electron transport inhibitors, the growth response of *C. vulgaris* cultures was rather different. The not comparable effective concentrations indicate that uptake and mode of action of each herbicide could be different in *C. vulgaris*. The growth rate of microalgal cultures at 48 and 96 h shows that both herbicides have inhibitory effects at the highest concentrations assayed (Table 1 and Table 2). Terbutryn is a more efficient inhibitor of growth of *C. vulgaris* cultures than isoproturon, since microalgal cultures exposed to isoproturon showed an EC_{50} value, after 96 h of treatment, twice that for the terbutryn-treated cultures(Fig. 2 and Fig. 5). *S*-Triazine herbicides, such as terbutryn, are considered one of the most efficient herbicides, at least when they are tested on freshwater algae (Abou-Waly et al., 1991), probably due to the presence of a methylthio group in position six of the triazine ring, that could tend to be linked with the relatively high inhibitory effect to algal cells (El-Dib et al., 1989). Moreover, differences in grade of toxicity can be associated with solubility of herbicides in lipids; it is well known that lipid-soluble substances easily pass into cells through the cell wall (Tang et al., 1998) and, that the sorption of the herbicide to algal cells is a prerequisite for its action at the chloroplast membrane. Isoproturon (log K_{ow} 2.25) can be considered a moderately lipophilic compound, but the triazine terbutryn (log K_{ow} 3.49) could be rapidly taken up, as a passive uptake, from the medium by *C. vulgaris* cells due to affinity of this molecule to the algal cell (Reddy and Locke, 1996). This hypothesis will agree with the results obtained in the present work.

In *C. vulgaris* cultures, the EC_{50} value after 96 h of herbicide exposure is higher than the EC_{50} after 48 h, for both herbicides assayed, suggesting that phytotoxicity of both herbicides at higher concentrations presents an acute action. Values obtained only after 48 h of toxic exposure can give a partial view of the facts occurring in the freshwater ecosystems.

After herbicide exposure, the cells of *C. vulgaris* try to increase the growth rate after a inhibitory growth period, being its duration proportional to the herbicide concentration (Table 1 and Table 2). Both growth curves and growth rates at 48 and 96 h show this phenomenon (Fig. 1 and Fig. 4). Therefore, the herbicides assayed may have an algistatic effect.

It is outstanding that 96 h EC_{50} values obtained for both herbicides are below the solubility limit in water for isoproturon (65 mg l^{-1}; 22 °C) and, terbutryn (22 mg l^{-1}; 20 °C) (Cremlyn, 1991), concentrations susceptible of affecting algal growth. Generally, PS II herbicide concentrations are below 0.1 µg l^{-1} (limit value for drinking waters, at least in european countries) (El Jay et al., 1997). However, several aquatic systems have been found to be contaminated with higher concentrations; for instance, herbicide concentrations higher than 2 µg l^{-1} have been found in several rivers of Europe (Beitz et al., 1994). This suggests that important effects would be caused on microalgae populations after herbicide application, leading to the disappearance of different species, as *C. vulgaris*, an ubiquous, constitutive and generally defined as a tolerant species of the plankton community in freshwater environments (Kasai and Hanazato, 1995).

The lowest concentrations assayed of the triazine herbicide causes an increase in the cellular density and growth rate of the test microalga after 96 h of exposure, not observed in the phenylurea herbicide-treated cultures (Table 2; Fig. 4). Stimulation effects are often recorded in algal bioassays with different species and different pesticides (El-Dib et al., 1991; Haglund, 1997; Franqueira et al., 1999). This stimulation of growth, obtained at the lowest terbutryn concentrations, indicates the ability of algal cells, and especially of *C. vulgaris*, to adapt and resist the inhibitory effect of herbicides. This may explain the increase observed in algae in herbicide-treated aquatic ecosystems and perhaps also in aquatic ecosystems subjected to toxicants (Shehata et al., 1997).

Dry weight and cell volume of *C. vulgaris* cells strongly increased in the presence of the highest concentrations of the two herbicides assayed, especially in the phenylurea cultures (Table 1 and Table 2).

These increases may be related to the growth inhibition at the highest herbicide concentrations. These two parameters showed a positive correlation for isoproturon and terbutryn (r=0.90 and 0.85, respectively). Photosynthesis-inhibiting herbicides may alter the overall bioenergetic status of the plant (Wilson et al., 2000), leading to the uncoupling of cell growth and reproductive processes, as reflected in the increase in the dry weight or the cell volume. Previous studies indicate that cell volume are correlated significantly with toxicity (Tang et al., 1998). It is important that this increase in dry weight and cell volume may influence the remaining parameters, pigment and protein content (Table 1 and Table 2; Fig. 3 and Fig. 6).

Since the herbicides assayed specifically exert its phytotoxic action at the photosystem II level, changes in microalgal chlorophyll content can be a reliable indicator of toxicity (Mayer et al., 1997); but the results suggest that these herbicides may affect chlorophyll and growth differently. In this study, at the concentrations of herbicides that provoke an inhibition of microalgal growth, chlorophyll content per cell is increased (Fig. 3 and Fig. 6).

Examples of triazine-induced increases of the algal chlorophyll content were reported in *Selenastrum capricornutum* by Mayer and Jensen (1995). It can be interpreted as a tolerance mechanism (François and Robinson, 1990). This process may result from a homeostatic mechanism triggered by the exposure to the herbicides. Responses such as the synthesis of thylakoid components are considered to be a general adaptation response to situations in which electron transport rate is strongly limited for photosynthesis (Behra et al., 1999).

The C/N ratio decreased as herbicides concentrations increased (Table 1 and Table 2).

As a result of the photosynthetic inhibition process, microalgal cells would not have enough energy for CO_2 fixation. This can be the reason why C content decreases in the cultures after 96 h of exposure to both herbicides assayed.

Since proteins were determined from the data of nitrogen content, the increase recorded in the cellular protein content may be provoked by the increase obtained in the cellular contents of N, and then can be explained by the increase of dry weight and cell volume (Table 1 and Table 2). An increase in protein content could be also related with a detoxification mechanism, for instance other herbicides as simazine have been shown to be detoxified by various microalgal species and subsequent binding to a protein (Kruglov, 1970).

Acknowledgements

This work was supported by a research project from Xunta de Galicia (Spain) (ref. PGIDT01MAM10302PR). C. Rioboo holds a fellowship from the Plan Nacional F.P.U., Ministerio de Educación y Cultura, Madrid, Spain. O. González holds a fellowship from the Conselleŕia da Presidencia, Xunta de Galicia (Spain). Thanks to the anonimous referees of this paper that contributed to improve it.

References

H. Abou-Waly, M.M. Abou-Setta, H.N. Nigg, L.L. Mallory
Growth response of freshwater algae Anabaena flos-aquae and Selenastrum capricornutum to atrazine and hexazinone herbicides
Bull. Environ. Contam. Toxicol., 46 (1991), pp. 223–229

R. Behra, G.P. Genoni, A.L. Joseph
Effects of atrazine on growth, photosynthesis, and between-strain variability in Scenedesmus subspicatus (Chlorophyceae)
Arch. Environ. Contam. Toxicol., 37 (1999), pp. 36–41

H. Beitz, H. Schmidt, F. Herzel
Occurrence, toxicological and ecotoxicological significance of pesticides in groundwater and surface waters
W. Ebing (Ed.), Pesticides in Ground and Surface Water, Springer-Verlag, New York (1994), pp. 279–314

T.E. Brown, F.L. Richardson, M.L. Vaughn

Development of red pigmentation in Chlorococcum wimmeri (Chlorophyta: Chlorococcales)
Phycologia, 6 (1967), pp. 167–184

P.-Y. Caux, L. Ménard, R.A. Kent
Comparative study of the effects of MCPA, butylate, atrazine, and cyanazine on Selenastrum capricornutum
Environ. Pollut., 92 (1996), pp. 219–225

R.J. Cremlyn
Agrochemicals: Preparation and Mode of ActionWiley, New York (1991)

M.A. El-Dib, S.A. Shehata, H.F. Abou-Waly
Response of freshwater alga Scenedesmus to triazine herbicides
Water Air Soil Poll., 48 (1989), pp. 307–316

M.A. El-Dib, A. Shehata, H.F. Abou-Waly
Response of freshwater algae (Scenedesmus sp.) to phenylurea herbicides
Water Air Soil Poll., 55 (1991), pp. 295–303

E. El Jay, J.M. Ducruet, J.C. Duval, J.P. Pelletier
A high-sensitivity chlorophyll fluorescence assay for monitoring herbicide inhibition of photosystem II in the chlorophyte Selenastrum capricornutum: comparison with effect on cell growth
Arch. Hydrobiol., 2 (1997), pp. 273–286

FAO/WHO, 1973. Amino acid content of foods and biological data on proteins, vol. 24, Rome, Italy.
François and Robinson, 1990
D.L. François, G.G.C. Robinson
Indices of triazine toxicity in Chlamydomonas geitleri Ettl
Aquat. Toxicol., 16 (1990), pp. 205–228

D. Franqueira, A. Cid, E. Torres, M. Orosa, C. Herrero
A comparison of the relative sensitivity of structural and functional cellular responses in the alga Chlamydomonas eugametos exposed to the herbicide paraquat
Arch. Environ. Contam. Toxicol., 36 (1999), pp. 264–269

E. Gnaiger, G. Bitterlich
Proximate biochemical composition and caloric content calculated from elemental CHN analysis: a stoichiometric concept
Oecologia, 62 (1984), pp. 289–298

K. Haglund
The use of algae in aquatic toxicity assesment
,in: F.E. Round, D.J. Chapman (Eds.), Progress in Phycological Research, 12Biopress, Bristol, UK (1997), pp. 181–212

F. Kasai, T. Hanazato
Effects of the triazine herbicide, simetryn, on freshwater plankton communities in experimental ponds
Environ. Pollut., 89 (1995), pp. 197–202

Y.V.a.E.I.P. Kruglov
Detoxification of simazine by microscope algae
Mikrobiologiya, 39 (1970), pp. 157–160

L. Larsen, S.R. Sorensen, J. Aamand
Mecroprop, isoproturon, and atrazine in and above a sandy aquifer: vertical distribution of mineralization potential
Environ. Sci. Technol., 34 (2000), pp. 2426–2430

H.K. Lichtenthaler
Chlorophylls and carotenoids: pigments of photosynthetic biomembranes
Methods Enzymol., 148 (1987), pp. 350–382

Mayer, P., Jensen, J.F., 1995. Factors affecting results of algal toxicity tests. In: Institute for Environmental Science and Technology, Technical University of Denmark, Lyngby.
Mayer et al., 1997
P. Mayer, R. Cuhel, N. Nyholm
A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests
Water Res., 31 (1997), pp. 2525–2531

P.V. McCormick, J.J. Cairns
Algae as indicators of environmental change
J. Appl. Phycol., 6 (1994), pp. 509–526

C. Nalewajko, M.M. Olaveson
Ecophysiological considerations in microalgal toxicity tests
P.G. Wells, K. Lee, C. Blaise (Eds.), Microscale Testing in Aquatic Toxicology. Advances, Techniques and Practice, CRC Press, Boca Raton, FL (1998), pp. 289–309

F. Pérés, D. Florin, T. Grollier, A. Feurtet-Mazel, M. Coste, F. Ribeyre, M. Ricard, A. Boudou
Effects of the phenylurea herbicide isoproturon on periphytic diatom communities in freshwater indoor microcosms
Environ. Pollut., 94 (1996), pp. 141–152

S. Reader, M. Marion, F. Denizeau
Flow cytometric analysis of the effects of tri-n-butyltin chloride on cytosolic free calcium and thiol levels in isolated rainbow trout hepatocytes
Toxicology, 80 (1993), pp. 117–129

K. Reddy, M.A. Locke
Molecular properties as descriptors of octanol–water partition coefficients of herbicides
Water Air Soil Poll., 86 (1996), pp. 389–405

F. Schanz, U. Zahler
Prediction of algal growth in batch cultures
Schweiz. Z. Hydrol., 43 (1981), pp. 103–113

H.M. Shapiro
Practical Flow Cytometry (3rd ed.)Wiley–Lyss, New York (1995)

S.A. Shehata, M.A. El-Dib, H.F. Abou-Waly
Effect of certain herbicides on the growth of freshwater algae
Water Air Soil Poll., 100 (1997), pp. 1–12

S. Singh, R.C. Kirkwood, G. Marshall
Effects of isoproturon on photosynthesis in susceptible and resistant biotypes of Phalaris minor and wheat
Weed Res., 37 (1997), pp. 315–324

B. Sosak-Swiderska, D. Tyrawska, B. Maslikowska
Microalgal ecotoxicity test with 3,4-dichloroaniline
Chemosphere, 37 (1998), pp. 2975–2982

J. Tang, K.D. Hoagland, B.D. Siegfried
Uptake and bioconcentration of atrazine by selected freshwater algae
Environ. Toxicol. Chem., 17 (1998), pp. 1085–1090

C. Tomlin
The Pesticide Manual. A World Compendium, The Bath Publisher, Bath (1994), p. 1341

S.D. Utting
Influence of nitrogen availability on the biochemical composition of three unicellular marine algae of commercial importance
Aquacult. Eng., 4 (1985), pp. 175–190

G.E. Walsh, R.G. Merril
Algal bioassays of industrial and energy process effluents
L.E. Shubert (Ed.), Algae as Ecological Indicators, Academic Press, London (1984), pp. 329–360

P.C. Wilson, T. Whitwell, S.J. Klaine
Phytotoxicity, uptake, and distribution of 14C-simazine in Acorus gramenius and Pontederia cordata
Weed Sci., 48 (2000), pp. 701–709

Corresponding author. Tel.: +34-981-167-000; fax: +34-981-167-065