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Optimal growth of *Dunaliella primolecta* in axenic conditions to assay herbicides

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Abstract

To develop an assay for herbicides in marine environments using microalgae, we have optimized the specie, cell culture media and physical conditions to obtain maximal cellular densities in a 96 well micro format to allow mass assays. We first surveyed several species of 7 unicellular eukaryotic algae genera (*Dunaliella, Tetraselmis, Chlorella, Ellipsoidon, Isochrysis, Nannochloropsis*, and *Phaeodactylum*) for vigorous in vitro axenic growth. Once the genus *Dunaliella* was selected, *Dunaliella primolecta* was preferred among 9 species (*bioculata, minuta, parva, peircei, polymorpha, primolecta, quartolecta, salina and tertiolecta*) because it showed the highest growth rates. The components (oligo elements, sugars, amino acids and vitamins) and conditions (light, CO_2, temperature) of the culture media were further optimized to obtain the highest cellular densities (up to 60×10^6 cells ml^{-1}) and the shortest cell cycle duration (~12 h) for *D. primolecta*. Then the toxicity of four representative herbicides, alloxydim, and sethoxydim (inhibitors of acetyl-coA carboxilase), metamitron (inhibitor of photosynthesis) and clopyralid (inhibitor of respiration), were assayed on the optimal culture conditions for *D. primolecta* during 96 h. The results showed that *D. primolecta* was susceptible to those herbicides in the following order: metamitron > sethoxydim > alloxydim. In contrast, clopyralid did not have any effects. Therefore, *D. primolecta* microcultures can be used to assay a large number of samples for the presence of herbicides under a saline environment.

Keywords: *Dunaliella*; Optimized culture; Microalgal assay; Herbicide assay

1. Introduction

Dunaliella are eukaryotic unicellular-biflagellated green microalgae (5–20 μm length by 1-10 μm wide) that belong to the genus *Chlorophyceae* and have a high protein content of 35-48% (Gibbs and Duffus, 1976). *Dunaliellas* have no rigid cell walls but a glycoprotein-cellulosic cell coat formed by 25–200 nm long fibrils (as stained by Indian ink at the optical microscope) or appearing as a thin electron opaque layer (at the electron microscope) (Gibbs and Duffus, 1976; Oliveira et al., 1980). *Dunaliellas* are mass cultured in saline lagoons worldwide to produce β-carotene at commercial scales (Ben-Amotz, 1999; Garcia-Gonzalez et al., 2000). *Dunaliella* species are known by its extreme adaptations to halo tolerance (0.05–5 M salt), pH (1–9), temperature (10–30 °C) (Gimmler and Weis, 1992) and light (10–1000 μE m^{-2} s^{-1}) (Aizawa and Miyachi, 1992).

All the above mentioned properties make *Dunaliellas* a potential optimal organism to detect herbicides in marine environments. The difficulties of axenic growth of any such organism to high enough cellular densities might be overcome by the use of a variety of conditions of culture while the lack of a true cell wall as a significant barrier to the permeability of the substances to be assayed is an obvious advantage among other possible microalgae. The halo

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tolerance of *Dunaliellas* allows to assay chemicals under different saline conditions thus mimicking the situations occurring in the fresh/saltwater interface. Laboratory studies with a series of chemicals tested with a biological model such as *Dunaliellas* might be useful in providing indication on the best or the worst of selected list of substances (Gaggi et al., 1995). The magnitude of effects will depend on the concentration and chemical properties of the substances to be assayed as well as its selective effects on the species exposed (Shehata et al., 1997).

The presence of chemical residues such as pesticides, because of their relatively high toxicity and direct usage with crops, has created an increasing awareness of their potential toxicological hazards in marine environments. Microalgae assays, such as the one proposed, would constitute a short-term, rapid, sensitive and relatively inexpensive procedure for estimating the toxicity in aquatic ecosystems (Sbrilli et al., 2005).

In the present study, *D. primolecta* was selected as the best marine microalgae grower in a defined and optimized axenic enriched medium. The *D. primolecta* optimized laboratory microculture was used to detect the impact of four representative herbicides (alloxydim and sethoxydim, inhibitors of acetyl carboxilase), metamitron (inhibitor of photosynthesis) and clopyralid (inhibitor of respiration). Other herbicides are presently under study.

2. Materials and methods

2.1. Microalgae species

Twelve different species of unicellular marine microalgae (Table 1) were kindly provided by Dr. Fábregas (Universidad de Santiago de Compostela, Spain). The *Dunaliella* species were obtained from the Culture Collection of Algae and Protozoa (CCAP, Cambridge, U.K.). The *Dunaliella* species included in this study were: *bioculata* (CCAP 19/4), *minuta* (CCAP 19/5), *parva* (CCAP 19/9), *peircei* (CCAP 19/2), *polymorpha* (CCAP 19/7a), *primolecta* (CCAP 11/34), *quartolecta*(CCAP 19/8), *salina* (CCAP 19/3) and *tertiolecta* (CCAP 19/6B).

2.2. Maintenance and growth

For maintenance, microalgae were holded at 17 °C, 0–5% CO_2 (as estimated with a CO_2 gas monitor, Fisher Bioblock Sci.Strasbourg, France), fluorescent light (Grolux, Silvania) at 20 μE m^{-2} s^{-1} (as estimated with a manual luminometer) and 14–10 h light–dark cycles. In the previous experiments, 3 different culture media in double distilled water were tested to obtain high concentrations of growing cells in axenic culture: MS, Murashige media (Sigma Che Co. St.Louis Ms), 30 mg ml^{-1} (~0.5 M NaCl) of marine salts (Acuamedic, Meersalz, Germany) and 50 μg ml^{-1} of gentamicin; MSTB, made by adding 0.3% of TB (12 g bactotriptone, 24 g bactoyeast and 8 ml glycerol per liter) to the MS and SAP, 30 mg ml^{-1} of marine salts, 5 mM Algal-1 (see later),

Table 1
Growth of several species of marine microalgae in enriched axenic culture media

Specie	Origin	Culture media		
		MS	MSTB	SAP
Dunaliella tertiolecta	CCAP	+	++	+++
Dunaliella parva	CCAP	+	++	+++
Dunaliella primolecta	CCAP	++	+++	++++
Tetraselmis suecica	Dr. Fábregas	±	+	±
Tetraselmis tetrahele	USC	±	+	±
Chlorella autotrophica	Dr. Fábregas	+	+	+
Ellipsoidon sp.	Dr. Fábregas	–	ND	ND
Isochrysis sp.	CCMP1324	–	ND	ND
Nannochloropsis atomus	Dr. Fábregas	±	–	–
Nannochloropsis israll	Dr. Fábregas	–	–	±
Nannochlorosis salina	CCMP 527	–	–	±
Phaeodactylum tricornutum	UTEX640	–	–	–

CCAP, Culture Collection of Algae and Protozoa (Cambridge, U.K.).
UTEX, University of Texas Culture Collection (Texas, USA).
USC, University of Southern California (California, USA).
CCPM, Center Culture of Marine Phytoplankton (ME, USA).
ND, not done.
–, no color.
±, light green.
+, green.
++ and +++, increasing green color intensities.

1.2 g l^{-1} peptone bactotryptone (pancreatic digest of caseins from Beckton-Dickinson, MD USA), 2,5 mg/ml of glucose, 20 mM Hepes, 5 mg l^{-1} phenol red, 50 μg ml^{-1} gentamicin, 100 mg ml^{-1} ampicillin and 2.5 μg ml^{-1} fungizone. All the media were filtered through 0.2 μm before use. The SA medium (see the composition in 3) was defined after the experimental evidence showed the optimal concentrations of the SAP medium and that neither bactotryptone nor glucose and phenol red were required (Fig. 1).

Final concentrations of the Algal-1 components at 5 mM of N were 5 mM KNO_3, 250 μM NaH_2PO_4, 66 μg l^{-1}, Na_2 EDTA, 50 μM Fe citrate, 2.5 μM, $ZnCl_2$, 2.5 μM $MnCl_2$, 2.5 μM $MoNa_2O_4$, 0.25 μM $CuSO_4$, 0.25 μM $CoCl_3$, 87.5 μg l^{-1} Thiamine, 12.5 μg l^{-1} Biotin, and 7.5 μg l^{-1} Cobalamine (Fabregas et al., 1985; Herrero et al., 1991).

For vigorous growth, microalgae were statically grown in 25 cm^2 cell culture flasks (Costar, The Netherlands) gassed with 20–30% CO_2 in air (flasks tightly closed), 150–300 μE m^{-2} s^{-1}, 25–28 °C and 14–10 light–dark photoperiod (Table 2). Cells were counted with a hemacytometer after being diluted 1:1 in 1% formaldehyde, 0.1% acetic acid and 0.1 M sodium phosphate pH 5.6. To estimate growth in 96-well micro plates in 100 μl of medium, absorbance at 405 nm were also used (Loeblich, 1982). A correlation between absorbance and number of *D. primolecta* cells was then established at several cell concentrations. In the conditions used 0.5 absorbance units at 405 nm corresponded to 12×10^6 cells per ml ($y =$; $37x–6.5$; $r^2 = 0.99$).

2.3. Chemicals tested

The following herbicides were tested: alloxydim, sethoxydim, clopyralid and metamitron (Table 3). Considering

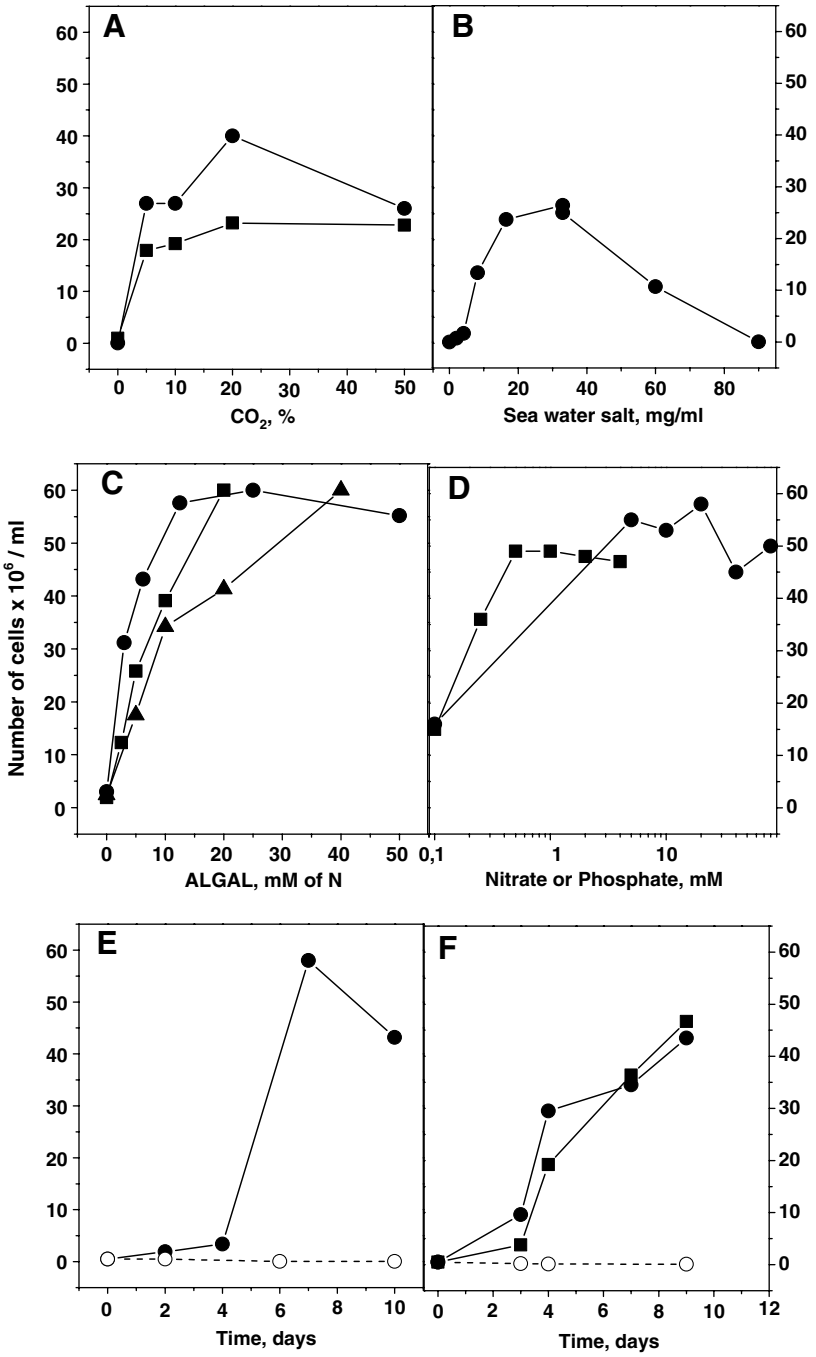


Fig. 1. Growth characteristics of *D. primolecta* in SAP medium. Cultures were performed in 25 cm^2 flasks gassed with CO_2. *D. primolecta* were inoculated at 0.5×10^6 cells ml^{-1} in 5 ml of SAP medium and grown in 30% CO_2, 150 μE m^{-2} s^{-1}, 30 mg ml^{-1} marine salts, 5 mM Algal-1, 1 mg ml^{-1} bactotryptone and 2.5 mg ml^{-1} glucose, unless otherwise indicated. A, Number of cells at different CO_2 concentrations after 7 days of incubation. ● and ■ , data from two different experiments. B, Number of cells at different concentrations of marine salts. C, Number of cells at different mM concentrations of nitrogen from Algal-1. ●, ■, and ▲, data from three different experiments. D, Number of cells at 20 mM of N of different Algal-1 mixtures made by including different concentrations of phosphate (■) and nitrate (●). E, Time course of growth at 20 (○) and 30% (●) of CO_2. F, Time course of growth at 20 (○), 200 (■) and 300 (●) μE m^{-2} s^{-1}.

the closed environments in which the test were carried out and the short duration of the experiment, no significant losses of the chemicals due to volatilization from the tested solutions were likely to occur. Each stock solution of herbicide was prepared in distilled water at a concentration 10–20-fold of the final concentration used for the culture. The used herbicides have a high enough solubility in water as to make possible those high concentrations (Tomlin, 1994).

2.4. D. primolecta toxicity test

Serial dilutions of herbicides to be assayed were made from the stock solutions (75 μl per well) in SA medium were pipetted into the wells of a 96-well plate. The inocolum in algal bioassay is prepared not more than 20–30 min prior to the beginning of the test, using algal cells harvested from two to four day stock culture. Then 75 μl of a *D. primolecta*

Table 2
Growth of several species of *Dunaliellas* in SAP medium

Catalogue number	Specie	Marine salts (mg/ml)	Growth relative absorbance
CCAP 19/4	*bioculata*	30	75.9 ± 8.6
CCAP 19/5	*minuta*	30	84.0 ± 6.2
CCAP 19/9	*parva*	30	80.3 ± 10.8
CCAP 19/2	*peircei*	60	7.0 ± 4.9
CCAP 19/7A	*polymorpha*	30	81.9 ± 8.1
CCAP 11/34	*primolecta*	30	100.0 ± 0.0
CCAP 19/8	*quartolecta*	30	79.8 ± 10.6
CCAP 19/3	*salina*	60	36.4 ± 26.7
CCAP 19/6B	*tertiolecta*	30	77.1 ± 16.7

*Average and standard deviations were calculated from three different experiments and expressed as relative absorbance by the formula: absorbance at 405 nm/absorbance at 405 nm of *D. primolecta* × 100.

culture at 5×10^6 cells ml^{-1}, as determined with a Neubauer hemocytometer, were added. The plates were agitated manually and briefly and then placed in a CO_2 impermeable high density polyethylene 17.5 × 22 cm bag (Esselte, Madrid, Spain), sealed and gassed with 30% CO_2 in air. The assay was carried out in an environmental chamber at 18–26°C under cool-white fluorescent lighting (200–400 μE m^{-2} s^{-1}), with 16–8 h light–dark photoperiod. For each substance to be assayed, the range of concentrations was determined by preliminary range-finding assays. The spiked concentrations of alloxydim, sethoxydim and clopyralid ranged from 0 to 1000 mg l^{-1}, and metamitron ranged from 0 to 100 mg l^{-1}. Three replicates were set up for each concentration and control.

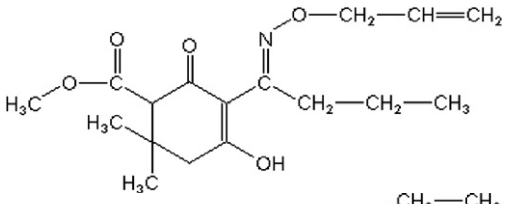
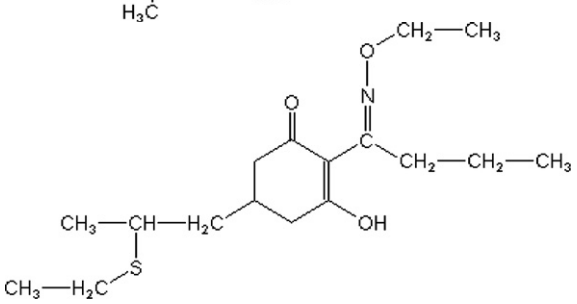
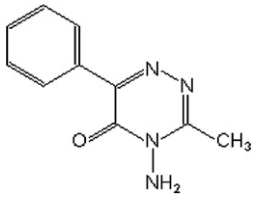
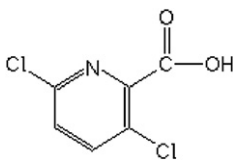
D. primolecta cellular density was estimated after 96 h of incubation by simply measuring the absorbance at 405 nm with a 96-well spectrophotometer after horizontal agitation of the plates (Labsystems ELISA reader).

2.5. Statistical analysis

An statistical analysis (ANOVA and Newman–Keuls Test) was carried out to determine if there were significant differences between the herbicide-exposed and control cells.

The principle of the assay is the determination of the concentration of test substance which results in a 50% reduction of growth within 96 h relative to the control (EC_{50}). The tested substance concentration which causes a 10% reduction of growth (EC_{10}) indicates a beginning inhibition (Nitschke et al., 1999). EC_{50} and EC_{10} values with 95% confidence limits were estimated by a non-linear

Table 3
Characteristics of the herbicides assayed in *D. primolecta* microcultures (Tomlin, 1994)

Herbicide	Solubility mg l^{-1} (temperature)	Chemical structure	Chemical class	Mode of action
Alloxydim	2×10^6 (30 °C)		Cyclohexanedione	Inhibits mitosis
Sethoxydim	25–4700 (pH 4–7) (25 °C)		Cyclohexanedione	Inhibits mitosis
Metamitron	1.8×10^3 (20 °C)		Triazinone	Inhibits photosynthesis
Clopyralid	9×10^3 (20 °C)		Pyridinecarboxylic acid	Cell elongation and respiration

regression method (Seefeldt equation). All statistical analyses were performed using the Statgraphics commercial software packages (Statgraphics, Advanced Regression Module Manual 1996, Rockville,MD, USA).

3. Results

3.1. Axenic culture media required to grow marine microalgae at high cell concentrations

For best reproducibility and sensibility, to be an assay, a defined cell culture media would be required to obtain the highest possible cellular density, the fastest growth and the best axenic conditions. Therefore, we first defined 3 different cell culture media (MS, MSTB and SAP) and assayed for the highest cell concentrations reached by the growth of 12 species of marine microalgae in those media. Most of 12 species of marine unicellular microalgae grew in synthetic marine salt water (30 mg l^{-1}) at $<1 \times 10^6$ cells ml^{-1}, depending on the specie. To increase cellular densities, the salt water was further enriched with peptone as a source of amino acids, vitamins and oligoelements while antibiotics were added to maintain axenic conditions. Table 1 shows the results obtained when 12 different species of microalgae were cultured in 3 different cell culture media (MS, MSTB and SAP).

Dunaliella, Tetraselmis and *Chlorella* in MS grew at cellular densities which were visible to the naked eye as green wells after 5–10 days of culture in 96-well plates (absorbance at 405 nm > 0.2). No evaporation was significant at the temperature used (20 °C).

To further increase those cellular densities, TB was added to the MS media (MSTB media). Although the final cellular concentrations obtained with MSTB were higher, contamination with bacteria and fungus was more frequent due to the high protein content. These results prompted to include ampicillin as a second antibacterial antibiotic and fungizone to slow down fungal contamination. Algal-1 (a commercial mixture of oligoelements and vitamins) was also added and peptone bactotryptone replaced the TB. Furthermore, Hepes and phenol red were added to stabilize the pH and to visualize the variations in pH, respectively. The resulting media, called SAP, was capable of sustaining vigorous growth of 3 *Dunaliella* species, especially that of *D. primolecta*. The rest of the microalgae species assayed in similar conditions (Table 1) were either not able to grow at similar rates nor to obtain similar densities even after 30 days of incubation (not shown). Nine *Dunaliella* sp were then obtained from the CCAP collection for further selection of species and optimization of growth.

3.2. Growth of 9 Dunaliella species in SAP medium

Table 2 shows the growth and additional species-specific marine salt requirements to be added to SAP to successfully grow all the 9 *Dunaliella* species. For instance, most *Dunaliella* grew with 30 mg ml^{-1} sea salts but *D. peircei* and *D. salina* required at least 60 mg ml^{-1} of marine salts. *D. primolecta* grew both faster and to final higher cellular concentrations than the rest of the *Dunaliella* species. *D. parva, D. quartolecta* and *D.tertiolecta* also grew vigorously. Because its excellent growth properties, *D. primolecta* was selected for further optimization studies.

3.3. Optimization of D. primolecta growth

D. primolecta cultures in 25 cm^2 cell culture bottles in SAP medium, gassed with 10% CO_2, illuminated at 150 μE m^{-2} s^{-1} and using an initial inoculum of 0.5×10^6 cells per ml, showed a slow growth phase of 4 days followed by an exponential phase of growth during the next 6 days. In those conditions, they reached a plateau at about $15–35 \times 10^6$ cells per ml (Fig. 1A). The final cellular density of *D. primolecta* could be increased up to 60×10^6 cells per ml by gassing the cultures with 20–30% CO_2 (Fig. 1E). Higher CO_2 concentrations did not increased the final cellular concentrations obtained and culture without CO_2 resulted in inhibition of any growth (Fig. 1E).

The concentration of marine salts was fixed at 30 mg ml^{-1} since optimal growth of *D. primolecta* was obtained between 20 and 40 mg ml^{-1} of marine salts (Fig. 1B).

Fig. 1C shows that the final concentration of *D. primolecta* was maximal at an amount of Algal-1 corresponding to 20 mM of its nitrogen content in the cell culture medium. The cultures with those Algal-1 concentrations reached a plateau at about 60×10^6 cells per ml when all other conditions were maintained optimal. Lower concentrations of Algal-1 resulted in lower plateau cell concentrations of *D. primolecta.* Omission of Algal-1 from the culture medium resulted in inhibition of growth under otherwise optimal conditions (not shown). Higher concentrations of Algal-1 did not increase further the final cellular concentrations (Fig. 1C).

Individual nitrate and phosphate components of the Algal-1 mixture where individually varied in the Algal-1 mixture and growth of *D. primolecta* tested in the corresponding media. Fig. 1D shows that both nitrate and phosphate were required for maximal growth at the optimal concentrations used in the Algal-1 mixture (~5 mM KNO_3 and ~250 μM NaH_2PO_4). Among all the rest of the Algal-1 components, only the removal of the EDTA caused strong inhibition in the growth of *D. primolecta* (data not shown), nevertheless, it was preferred to include all the components of the Algal-1 mixture for further experiments because of commercial availability of the mixture. Bactotriptone and sacarose could be omitted without any detectable changes in the final cellular concentrations and/or division rates (data not shown) and it was chosen to do so to further decrease the probabilities of culture contamination. On the other hand, concentrations of Gentamicin, Ampicillin and Fungizone could be increased 2–4 fold without adversely affect the *D. primolecta* cultures, thus maximizing protection against contamination (data

Table 4
Parameters of regression equations that describe the relationships between increasing rates of herbicides and growth of *D. primolecta*

Herbicides	*d* cm	*c* cm	B [cm/(mg l^{-1})]	EC_{10} (mg l^{-1})	EC_{50} (mg l^{-1})	R^2 (%)
Alloxydim[a]	2.09	−1.28	1.29	177.20	973.20	87.1
Sethoxydim[a]	1.95	0.072	1.66	23.32	87.63	87.6
Metamitron[a]	2.12	0.103	1.67	0.76	2.87	89.6
Clopyralid	Not adjusted to a regression equation. No inhibitory effect					

[a] Regression equation by Seefeldt model ($y = c + ((d-c)/(1 + \exp(b^*(\log(\text{doses}+1)-\log(EC_{50}+1)))))$).

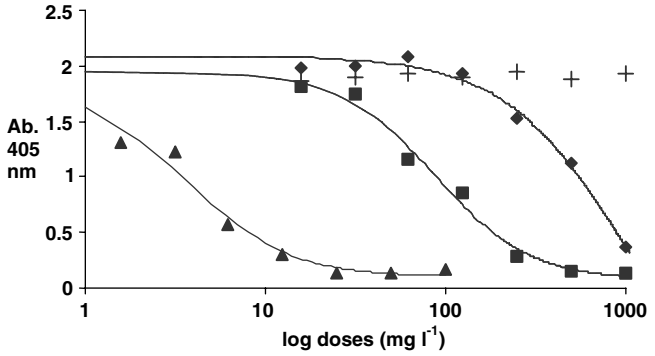


Fig. 2. Dose–response relationships of *D. primolecta* growth to the addition of herbicides. *D. primolecta* was grown in 96-well plates in the presence of different concentrations of alloxydim (◆), sethoxydim (■), metamitron (▲) and clopyralid (+). After 96 h, the Absorbance (Ab) at 405 nm of the cultures was measured in an ELISA reader (0.5 absorbance units at 405 nm corresponded to 12×10^6 cells ml^{-1}). Three replicates were set up for each concentration and control. Averages are represented in the figure.

not shown). The phenol red was also omitted to avoid interferences with the estimations of absorbance at 405 nm.

Taking all the above mentioned observations in consideration, the SA medium was finally defined as: 30 mg ml^{-1} of marine salts, 20 mM Hepes, 20 mM Algal-1, 100 μg ml^{-1} gentamicin, 200 mg ml^{-1} ampicillin and 5 μg ml^{-1} fungizone.

Under those conditions the time course of *D. primolecta* in flasks of 25 cm^2 and at 150 μE m^{-2} s^{-1} showed a lag period of about 4 days and a sharp increase between 4 and 6 days. However, the lag period could be eliminated and growth resulted almost linear from the beginning of the culture when using higher light intensities such as 300 μE m^{-2} s^{-1} (Fig. 1F), thus lowering the required time to perform the assay.

3.4. *D. primolecta* growth in the presence of herbicides

Preliminary results showed that *D. primolecta* exhibited different sensitivities to the presence of several kinds of herbicides in the culture medium. Thus a reduction in their growth rates and final cell concentrations were observed with an increase of some herbicide concentrations. The herbicides alloxydim and sethoxydim (inhibitors of the acetyl-CoA carboxylase), metamitron (inhibitor of photosynthesis) and clopyralid (inhibitor of respiration), were chosen (Table 3) for further studies, as examples of the different types of *D. primolecta* responses found.

D. primolecta growth inhibition in the presence of different concentrations of the selected herbicides was calculated by adjusting the dose–response data with regression equations (Table 4). Dose–response data showed that the toxicity responses of *D. primolecta* increased in the following order metamitron > sethoxydim > alloxydim > clopyralid (Fig. 2).

Doses of 177, 23 and 0.76 mg l^{-1} of alloxydim, sethoxydim and metamitron, respectively, produced the beginning of growth inhibition (EC_{10}) while clopyralid did not produced any effects at the dosages assayed (Table 4).

The EC_{50} of the herbicides that block "de novo" synthesis of fatty acids by inhibiting the activity of acetyl-CoA carboxylase were 973 and 87 mg l^{-1} of alloxydim and sethoxydim, respectively. The EC_{50} of metamitron (inhibitor of photosynthesis) was only 2.9 mg l^{-1}. *D. primolecta* was resistant to clopyralid at the range of concentrations assayed (Fig. 2).

4. Discussion

Marine microalgae will grow axenically in culture by using minimal media containing synthetic salts. However, growth under those conditions will often result on cellular densities around or below 10^6 cells ml^{-1} (Berges and Franklin, 2001). To further increase cellular densities, additional sources of amino acids and vitamins (for instance, Algal-1) and/or other mixtures containing defined components (found in protein hydrolisates such as yeast extracts, bactotriptone or peptone) should be added.

In addition, the inclusion of gentamicin–ampicillin and fungizone reduces the chances of contamination with bacteria and fungus, respectively. In the presence of optimal concentrations of Algal-1 (Fabregas et al., 1985; Herrero et al., 1991) and antibiotics (SA media), we could obtain cellular densities of *D. primolectas* $> 60 \times 10^6$ cells ml^{-1}. Other densities reported earlier for *D. salina* were: $\sim 1.0 \times 10^6$ cells ml^{-1} (Avron, 1992; Berges and Falkowski, 1998) in enriched salt sea water, $\sim 3 \times 10^6$ cells l^{-1} (Golldack et al., 1994; Golldack et al., 1995) or $\sim 9 \times 10^6$ (Geng et al., 2003) in defined cell culture media containing vitamins. To our knowledge, the highest density reported was $\sim 25 \times 10^6$ (Jin et al., 2001). On the other hand, a rate of ~2 duplications per day was obtained, which compares well with ~1.5 duplications per day obtained in a comparative study of several species of *Dunaliella* (Cifuentes et al., 2001).

Axenic cultures and optimal growth conditions of any unicellular marine microalgae would favour assay of substances such as herbicides. Recent results showed higher cellular concentrations would reflect the optimal conditions for grow and that those will offer the best sensibility to assay herbicides in *D. primolecta* (preliminary results). The wide application of herbicides in plant cultures world

wide could produce adverse effects on both fresh and marine aquatic ecosystems, due to their presence on marine coastal environments, in areas nearby agricultural fields. The results obtained confirmed that *D. primolecta* is a susceptible specie capable to detect the presence of some of the herbicides assayed, in the following order: metamitron > sethoxydim > alloxydim > clopyralid.

The EC_{50} values at 96 h of herbicides of the family that block "de novo" synthesis of fatty acids by inhibiting the activity of acetyl-CoA carboxylase were 973 and 87 mg l^{-1}. Those values were higher than the ones previously detected by using *Chlorella* with herbicides of the same family (Ma et al., 2002). The same authors found that the EC_{50} values of the photosynthesis-inhibiting herbicides were the lowest among the herbicides tested in *Chlorella*. On the other hand, the EC_{50} value at 96 h, obtained for metamitron (inhibitor of photosynthesis) was only 2.9 mg l^{-1}. This value was lower than those previously reported by using *Chlorella* with herbicides of the photosynthetic family (Ma et al., 2002) and similar to those obtained by Nitschke (Nitschke et al., 1999) by using *Scenedesmus subspicatus*.

On the other hand, if confirmed by further work, the resistance of *D. primolecta* to clopyralid could be used to investigate the mechanism of resistance of *D. primolecta* because exist possibilities to isolate a resistance gene.

The results obtained confirmed that microalgae cultures are useful indicators of potential pollution since they respond with inhibition of growth at low concentrations of substances such as herbicides (Sbrilli et al., 2005). Therefore, microalgae toxicity testing by growth inhibition can prove particularly advantageous as an initial screen for pesticide assessment (St-Laurent et al., 1992; Sabater et al., 2002). Results confirmed that the laboratory microculture approach for measuring the toxicity of chemicals is probably most appropriated for producing ranks of more or less susceptible microalgae and more or less hazardous chemicals, particularly when the mechanism of action of the toxic is known (Gaggi et al., 1995). In this sense, the laboratory studies described here with the representative herbicides tested may provide relevant data about the susceptibility of *D. primolecta*, in the form of ranks, especially with different chemical species. Therefore, microalgae assays, such as the one proposed here, might constitute a short-term, rapid, sensitive and relatively inexpensive procedure for estimating the toxicity in marine aquatic ecosystems (Sbrilli et al., 2005).

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