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EFFECTS OF ALLELOCHEMICAL 2-BENZOXAZOLINONE ON GROWTH, PIGMENT CONTENT AND CELL APPEARANCE OF *Tetraselmis suecica* (Kylin) Butch.

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Keywords:

ABSTRACT

2-benzoxazolinone (BOA) allelopathic activity to the prasinophyte *Tetraselmis suecica* has been studied on the basis of the effect of this allelochemical on growth, pigment content and cell appearance. Toxicity was evaluated on the microalga in a 96 h test where *T. suecica* was exposed to 10^{-5}, 10^{-4} and 10^{-3} M of BOA. Results showed a concentration-dependent effect of the studied factors. Severe effects on pigment content and growth parameters were manifested by the highest BOA concentration assayed: increase in total chlorophyll and carotenoids per cell of about 3 fold and reductions in growth rates of the 77.8 % have been revealed after 96 h of exposition. BOA 10^{-3} M also caused morphology changes in the cell that presented a higher area and a largest elliptical form. Ecological implications and suitability of *T. suecica* in allelopathic test are discussed.

INTRODUCTION

Photosynthesis is considered the primary metabolic process in every ecosystem and can be affected by a wide range of direct or indirect mechanisms (Einhellig, 1986). Photosynthesis is sensitive to different types of biotic and abiotic environmental conditions, such as light, temperature, pathogen attack, allelopathic compounds, etc. Therefore photosynthetic organisms must adapt to unfavourable conditions in their environment to optimise and preserve the function of the photosynthetic apparatus (Masojídek *et al.*, 2000).

A correlation between photosynthetic alteration and the action of some allelochemical compounds was showed in previous works (Einhellig, 1986; Hejl *et al.*, 1993), being the disruption of electron transport chain one of the most usual ways for affecting photosynthesis by allelochemical compounds (St. John, 1982; Al-Khatib *et al.*, 1992; Nimbal *et al.*, 1996; González *et al.*, 1998). But effects on photosynthesis can be also the result of an alteration in chlorophyll biosynthesis or in chlorophyll degradation pathway, inhibition of carotenoid biosynthesis (which can act as protective pigments against free radicals in chloroplast), or an inhibition of the enzyme protoporphyrinogen oxidase, which leads to chlorophyll biosynthesis (Henriksen *et al.*, 2002; Müller *et al.*, 2001; Masura *et al.*, 2002; Yang *et al.*, 2002).

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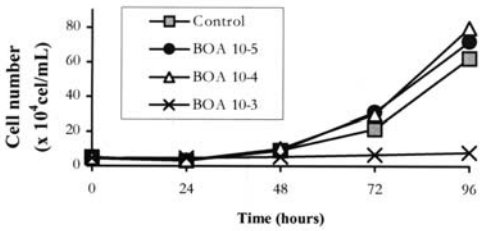

Figure 1.
Growth of Tetraselmis suecica *at different BOA concentrations*

Mersie and Singh in 1993 found a direct relation between the application of some phenolic acids (vanillic, chlorogenic, ferulic and *p*-coumaric) on *Abutilon theophrasti* Medik and an inhibition of the photosynthetic rate. They proposed this physiological effect to be the potential cause for the evident plant growth diminution. In a recent work, Yang *et al.* (2002) carried out an experiment to test the inhibitory effect of three allelopathic phenolics on the chlorophyll biosynthetic pathway of *Oryza sativa* seedlings. They found that accumulation of chlorophyll and porphyrin contents were more inhibited when the phenolic concentrations increased, suggesting an alteration in biosynthetic pathway by effects on chlorophyll precursors. In most of these allelopathic situations, decreases in photosynthesis have been correlated with decreases in chlorophyll content (Rama Devi *et al.*, 1997).

However, little is known about the toxicity of these compounds against aquatic flora, despite the importance of aquatic plants in the ecosystem function, considering that alterations in species composition of a marine community as a result of toxic stress may affect the structure and the functioning of the whole ecosystem.

Toxicity of the allelochemical 2-benzoxazolinone was assessed on the unicellular green alga, *Tetraselmis suecica* (Kylin) Butch, which is a phytoplankton species largely distributed throughout the world and used in aquaculture and toxicity assays, to evaluate the effects of sediment toxicity (Matthiessen *et al.*, 1998) and in bioaccumulation test because is able to accumulate large amounts of metals like cadmium from the contaminated medium.

2-benzoxazolinone (BOA) is a cyclic hydroxamic acid derived from 1,4-benzoxazin-one structures which are secondary metabolites commonly occurring in both cultivated and wild Gramineae (Zúñiga *et al.*, 1983; Niemeyer, 1988). Hydroxamic acids have been isolated and reported to play an important role in grass allelopathy (Barnes and Putnam, 1987; Chase *et al.*, 1991; Blum *et al.*, 1992; Friebe *et al.*, 1997; Kato-Noguchi, 2000). All of them show different biological activities (Niemeyer, 1988). These metabolites were discovered researching resistance of overwintering rye to *Fusarium nivale* (Virtanen & Hietala, 1955) and in a study of resistance of young maize plants to the European corn borer (Loomis *et al.*, 1957). In the last years, several works have reported effects of BOA on plant growth, focusing the study on growth and germination rates of the tested organism as well as on energy-linked reactions. But our knowledge of action mechanisms in plant physiology is very limited, especially when considering algal metabolism.

Therefore, the aim of the present study was to examine the changes in growth and photosynthetic pigments during exposure of the unicellular alga *Tetraselmis suecica* to different solutions of the allelochemical BOA.

MATERIAL AND METHODS

Plant material and culture conditions.

The unicellular prasinophyte *Tetraselmis suecica* was supplied by the Department of Biochemistry, Genetic and Immunology of the University of Vigo. Microalgae was grown photoautotrophycally in filtered and sterilised natural seawater medium (20 min at 121 ºC) enriched with f/2 nutrients (Guillard and Ryther, 1962). Cells were grown in 500 mL Erlenmeyer flasks containing 250 mL of medium at 20 ºC under a 12 h photoperiod (irradiance 80 $\mu E\ m^{-2}\ s^{-1}$) for 96 h. Initial cell density was 50 x 10^4cells/mL. Salinity of seawater was 37‰ and the initial pH of the culture was 8.1 ± 0.1. All cultures were carried out axenically.

Test solutions of BOA (Aldrich, 98 % purity) were prepared to get a final concentration in the medium of 10^{-5}, 10^{-4} and 10^{-3} M. BOA was previously diluted in

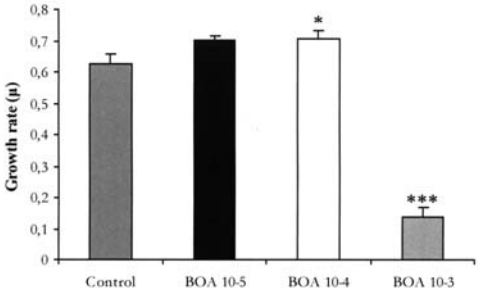

Figure 2.
Effect of increasing BOA concentrations on the growth rate of Tetraselmis suecica *after 96 h BOA exposition. Asterisks mean significant differences with respect to the control: *, p≤ 0.05 and ***, p≤ 0.001.*

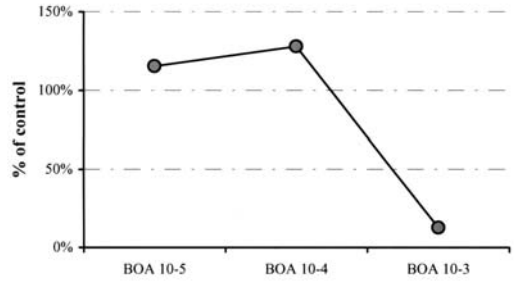

Figure 3.
Growth response of Tetraselmis suecica *after 96 h exposition to different BOA concentrations.*

methanol (0.1 %; v/v). Control was amended with the same quantity of methanol without BOA. Cultures were shaken gently everyday to ensure homogeneous exposure to the allelochemical. Four replicates were used for each treatment.

Growth rate.

Growth densities were determined with a Thoma (Marienfeld, Germany) hemocytometer and a Nikon, alphaphot-2 (Japan) light microscope at an amplification of 200 x. After 24, 48, 72 and 96 h of BOA exposition, 4 mL of cell cultures were collected and used to daily measurements of microalgae growth by counting the cells in 5% Lugol solution.

Growth rate (μ), expressed in day^{-1} (d^{-1}) was calculated by the usual formula $\mu = (\ln N_t - \ln N_0) / (t-t_0)$, where N is cellular density at time t after allelochemical exposure. Time is expressed in days. In order to express toxicity, data were shown as IC_{50} calculated by lineal interpolation.

Cell morphology.

To facilitate the study of the action of BOA on the morphology of *T. suecica cells*, samples of this microalgae exposed an unexposed to 10^{-3} M of BOA for 96 h were examined with an image analyser (Olympus MicroImage 3.01). Parameters analysed were area and shape. Area was the cell surface for samples from treatment and control and shape was the ratio between main and minor axis of the cell. Then, values in close proximity to 1 mean the cell is spherical. Data from 300 cells were recorded for treatment and control.

Pigment extraction and quantification of pigment contents.

20 mL from the different cultures were filtered daily (Albet FV-C) and filters frozen at -80 ºC prior analysis. Chlorophyll *a* and *b* were extracted from the cells in the filter in 3 mL of cold methanol. After cell breakage by sonication (Branson 1200, U.S.A.), suspension was filtered (Albet FV-F, 0.7 μm) to remove cell and former filter debris.

For HPLC analysis, a Hewlett-Packard Series 1100 (Hewlett-Packard, Palo Alto, Estados Unidos) equipped with a fluorescence detector (Hewlett-Packard 1046) and an Eclipse® XDB-C8 column (150 x 4.6 mm, 5 μm particle size, 80 Å pore size). The chromatographic conditions have been previously described by Garrido and Zapata, 1991. 1 mL of methanol extract was mixed with 0.2 mL of water to avoid shape distortion of earlier eluting peaks (Garrido and Zapata, 1991). Then 100 μL were injected. Pigments were quantified using external standards. Chlorophylls *a* and *b* were obtained from Sigma-Aldrich.

Carotenoid content was assessed spectrophotometrically from the methanol extract using the extinction coefficients according to Wellburn (1994). Optical densities were measured with a UV/Vis Diode-Array Spectrophotometer, (WPA, United Kingdom). To avoid pigment degradation, each sample was extracted immediately before its analysis and all the operations were performed under subdued light.

Statistical analysis.

ANOVA was used to detect significant variation in sample means due to treatment effects. Homogeneity of variance of the data was tested using a Levene test. If this assumption failed ($p \leq 0.05$) then a Kruskal-Wallis rank order nonparametric ANOVA analogue was used. Significant differences in sample means were evaluated with the LSD test.

RESULTS

Growth measurements.

The effect of different BOA concentrations on growth of Tetraselmis suecica cells over a 96 h exposure period is shown in figure 1. The data showed significant inhibition of *T. suecica* growth at 10^{-3} M BOA concentration. Minor BOA concentrations significantly enhanced growth after 2 days for 10^{-4} M and 3 days for 10^{-5} M (Table 1). Control and treatment cultures revealed significant differences among them after 24 h, but the temporal analysis for each of the treatments did not show differences between times 0 and 24 h.

T. suecica growth rates (μ) calculated from day 0 to day 4 are shown in Fig. 2. 10^{-3} M BOA concentration led to a significant reduction of growth ($\mu = 0.14$), while in the other assayed concentrations there is a tendency to increase the growth rate that is correlated with the increase of the concentration. So, we obtain 0.63, 0.70 and 0.71 values for 0, 10^{-5} and 10^{-4} M BOA respectively.

Along the 96 h of experiment, the growth decreased in the cultures with the highest concentration of BOA in a significant way with respect to the control (Fig. 3). Strong reduction of 87% was uncovered for concentration 10^{-3} M and a slight linear increase was detected for 10^{-5} and 10^{-4} M with values 15 and 28 % in excess of the control. IC_{50} value was calculated based on day 4 concentrations by lineal interpolation from figure 3. In this case, the IC_{50} was $7.08 \cdot 10^{-4}$ M BOA.

Cell morphology.

Data related to appearance (area and shape) from cultures exposed to 10^{-3} M of BOA and controls for 96 h are recorded in figure 4. Cells exposed to the

Table 1. *Differences in growth of Tetraselmis suecica among different BOA concentrations: 0, 10-5, 10-4 and 10-3 M as a function of period of incubation. Asterisks mean significative inhibition and spots significative stimulation: *, p = 0,05; **, p = 0,01; ***, p = 0,001; •, p = 0,05; ••, p = 0,01, •••, p = 0,001.*

Treatments		0 h	24 h	48 h	72 h	96 h
Control	BOA 10^{-5}	n.s.	n.s.	n.s.	***	***
	BOA 10^{-4}	n.s.	n.s.	*	***	***
	BOA 10^{-3}	n.s.	***	•••	•••	•••
BOA 10-5	Control	n.s.	n.s.	n.s.	•••	•••
	BOA 10^{-4}	n.s.	n.s.	*	n.s.	**
	BOA 10^{-3}	n.s.	***	•••	•••	•••
BOA 10-4	Control	n.s.	n.s.	•	•••	•••
	BOA 10^{-5}	n.s.	n.s.	•	n.s.	••
	BOA 10^{-3}	n.s.	***	•••	•••	•••
BOA 10-3	Control	n.s.	•••	***	***	***
	BOA 10^{-5}	n.s.	•••	***	***	***
	BOA 10^{-4}	n.s.	•••	***	***	***

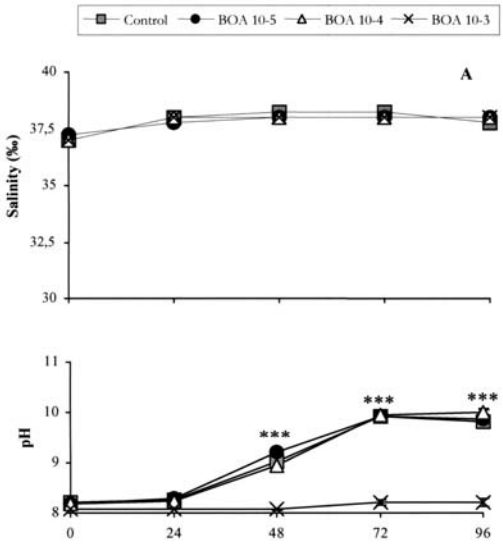

Figure 4.
BOA Effect on cell morphology of Tetraselmis suecica *after 96 h of exposure to the allelochemical. Asterisks mean significant differences with respect to the initial culture* ***, *p≤0.001.*

allelochemical showed a higher area and a largest elliptical form (30.59 and 7.69 % respectively) than cells growing without allelochemical. Means were significantly different at level of 1 ‰.

Salinity and pH.

Initial salinity of the cultures was 37 ‰ and did not vary significantly along the experiment (Fig. 5 A). pH-values depicted in figure 5 B, show the pH evolution for the treatments. Initial values were 8.08 for BOA concentration 10^{-3} M and around 8.15 for the other treatments. pH in control and lowest concentration showed a significant smooth increase ($p \leq 0.05$) during the growth phase, from 24 to 72 h. The highest concentration of BOA induced a significant pH uprising from 48 to 72 h, showing an increase delay evolution compared to the control. From 72 to 96 h all treatments showed a steady state in pH.

Pigments content.

Chlorophyll levels were normalized per cell. Figure 6 shows chlorophyll *a* and *b* contents per cell of *T. suecica*. Temporal progress of the chlorophyll content in the cultures with lower concentration of BOA (0, 10^{-5} and 10^{-4} M) were very similar and showed, after an initial increase within the first 48 h, a clear reduction in chlorophyll *a* and *b* production per cell after 48 and 72 h respectively. 10^{-3} M BOA showed *a* different pattern. Evolution of chlorophyll a content in those cultures did not show a reduction after 48 h and continuously increased the concentration up to 5 times initial chlorophyll content and about 4 fold more chlorophyll *a* per cell than the other cultures. Chlorophyll *b* showed a similar tendency but, by contrary, did not show remarkable differences in the period of 72 h.

Not normalised data per cell showed increase of variability after 24 h, and sometimes the statistical analysis did not distinguish among samples despite clear differences in the mean values. Bearing this in mind, the most notable fact is that 24 h after beginning incubation the cultures with 10^{-3} M BOA have more chlorophyll *a* and *b* than the control ($p \leq 0.01$ and $p \leq 0.05$ for chlorophyll *a* and *b* respectively). After this point, 10^{-3} M BOA induced a slight increase in

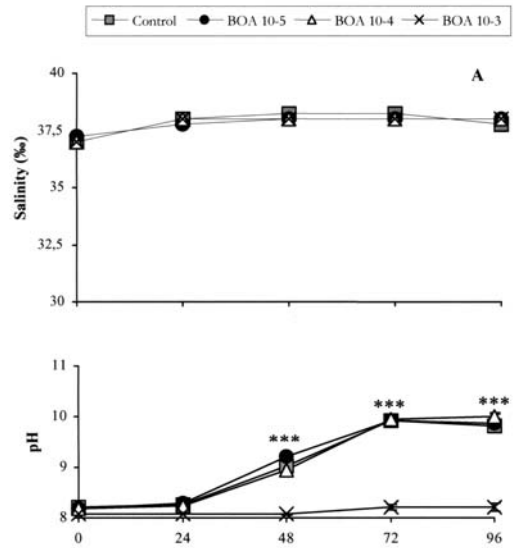

Figure 5.
Evolution of salinity (A) and pH values (B) in cultures of Tetraselmis suecica *growing in presence of different BOA concentrations. Asterisks mean significant differences with respect to time 0:* ***, *p≤0.001.*

Table 2.
Chlorophyll per volume unit of Tetraselmis suecica *cultivated on different BOA concentrations: 0, 10^{-5}, 10^{-4} and 10^{-3} M as a function of period of incubation. Asterisks mean significative inhibition and spots significative stimulation:*
**, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; •, p ≤ 0.05; ••, p ≤ 0.01, •••, p = 0.001.*

Total Chlorophyll (µg/mL)	0	24	48	72	96
Control	0.047 ± 0.007	0.052 ± 0.004	0.423 ± 0.048	0.677 ± 0.131	1.142 ± 0.145
BOA 10^{-5}	0.053 ± 0,07	0.047 ± 0.001	0.351 ± 0.040	1.028 ± 0.093	1.574 ± 0.088
	n.s.	n.s.	n.s.	•	••
BOA 10^{-4}	0.046 ± 0.005	0.067 ± 0.012	0.448 ± 0.063	1.145 ± 0.077	1.568 ± 0.149
	n.s.	n.s.	n.s.	••	••
BOA 10^{-3}	0.063 ± 0.005	0.087 ± 0.007	0.166 ± 0.005	0.393 ± 0.076	0.527 ± 0.136
	n.s.	••	**	n.s.	**

chlorophyll content whilst the other cultures experimented a dramatic increase of chlorophyll, especially in the cultures with BOA 10^{-4} and 10^{-5} that shown stimulatory differences with respect to the control ($p \leq 0.05$) (Table 2).

Figure 7 shows the effect of BOA at 96 h after beginning of the experiment, then the final effect on chlorophyll content. Comparison between normalized and not normalized data was established. Suspensions

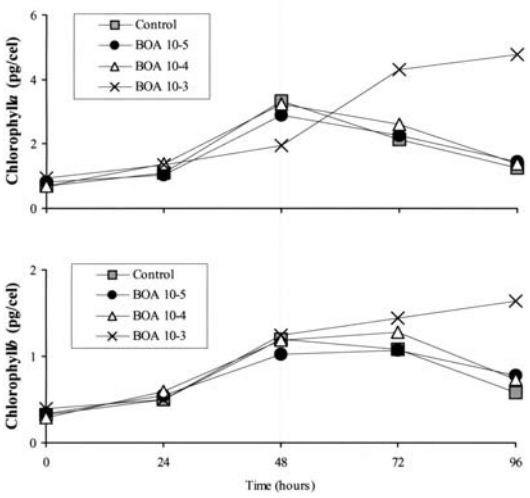

Figure 6.
Evolution of chlorophyll a *and* b *content per cell in Tetraselmis suecica exposed to different concentrations of BOA. Asterisks mean: *, p≤0.05 and ** p≤0.01.*

with a high growth level, control and 10^{-5} and 10^{-4} M BOA produced the same chlorophyll content per cell than control and the effect is similar in total chlorophyll per unit suspension volume. Highlighted result is showed for treatment 10^{-3} M where the chlorophyll content per cell is significantly enhanced ($p \leq 0.01$) and chlorophyll in the culture declined dramatically ($p \leq 0.01$).

Carotenoids final concentration per cell and volume of culture is showed in figure 8. Control, BOA 10^{-5} and 10^{-4} M brought about the same quantity of carotenoids per cell and the same result is showed for the concentration per volume. On the contrary, the samples with BOA 10^{-3} M showed significant differences with respect to the control and its standardised concentration increased significantly ($p \leq 0.001$) whereas the quantity of carotenoids per volume decrease significantly ($p \leq 0.01$) with respect to the control.

DISCUSSION

BOA has been reported as one of the allelochemicals affecting photosystem II (Reigosa *et al.*, 2001). Beginning of chlorosis appeared as one of the characteristic symptoms produced by BOA in light bioassays (Barnes and Putnam, 1987), resembling that of several commercial photosynthetic-inhibitor herbicides. Therefore, different concentrations of BOA were bioassayed on different physiological parameters of *Tetraselmis suecica* including photosynthetic pigments.

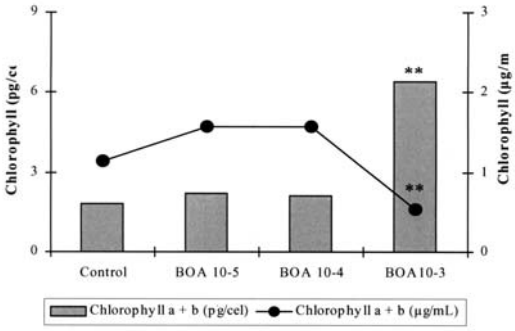

Figure 7.
Total chlorophyll concentration per volume and cell of the Tetraselmis suecica *suspension grown for 96 h in the presence of BOA. Asterisks mean: ** p≤0.01.*

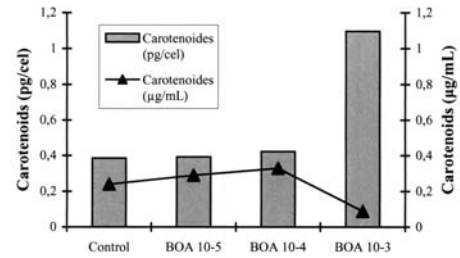

Figure 8.
Total carotenoids concentration per volume and cell of the Tetraselmis suecica *suspension grown for 96 h in the presence of BOA. Asterisks mean: ** p≤0.01 and *** p≤0.001.*

Bioassay duration is short enough to detect sensitivity of *Tetraselmis suecica* to the allelochemical without possibility of adaptation processes (Soukupová, *et al.*, 1999).

Interaction of allelochemicals on growth is dependent on both concentration and state of the target plant (Reigosa *et al.*, 1999). The suspensions exposed to BOA 10^{-3} M suffered a slow increase in the number of cells showed at 48 h growing. However, at the end of the experiment those cultures reflected a significantly different inhibition of the control meanwhile the cultures exposed to minor concentrations of BOA showed a greatest growth respect to the control.

The size of *T. suecica* cells after 96 h exposition to the strongest BOA concentration showed an increase of around 30% compared with the control. This increase was reflected as area enlargements as well as changes in the cell morphology. These changes are similar to the morphological changes suffered by *T. suecica* cells after exposition to high copper concentrations, although in this case the cells appeared to have a more spherical form (Nassiri *et al.*, 1996). The demonstrated ability of this green microalga to ameliorate toxicity under heavy metal exposition is based on its capacity to detoxify copper, cadmium and other compounds. Perhaps the absence of effects under low BOA concentrations could be explained as the result of detoxification processes occurring into the algal metabolism.

Stimulatory effects at lower concentrations of BOA (10^{-5} and 10^{-4} M) may be due either to the culture ability to use BOA as carbon resource or perhaps BOA has the capacity in the media to chelate iron being the most of them bound as complexes (Tipton and Buell, 1970). The iron complex of the cyclic hydroxamic acids is a source of iron available for plants. The complex is taken up through the membrane into the cell, where the iron increases the chlorophyll content and moderates the chlorosis (Pethô, 2002). Iron deficient crops take up more complexes, their metabolism is increased compared to those grown in culture solution containing iron. It is possible to assume that the inhibitory effect at high concentrations is related to the excess of BOA that has an allelopathic role by inhibiting the cell growth.

IC_{50} was determined using growth, parameter often used in toxicity tests because it is sensitive enough reliable, simple and easy to run (Wang, 1990). *T. suecica* was more sensitive in this bioassay to BOA than other microorganisms. This is supported by the value obtained for IC_{50} using growth that was lower than the obtained by Barnes and Putnam (1987) on *Chlamydomonas rheinhardtii*. Inhibitory concentration was estimated graphically using growth data from 96 h (Torres *et al.*, 2000). IC values could also have been calculated based on 72 or 48 h, where there are significantly differences between inhibitory concentrations and control. So, the values would have been much lower (Verdisson *et al.*, 2001) and thus the toxicity of the allelochemical considered much higher.

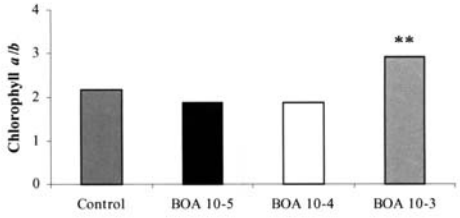

Figure 9.
Effect of different BOA concentrations on chlorophyll a *to chlorophyll* b *ratio after 96 hours of exposure. Asterisks mean significant differences with respect to the control: **, p≤0.01.*

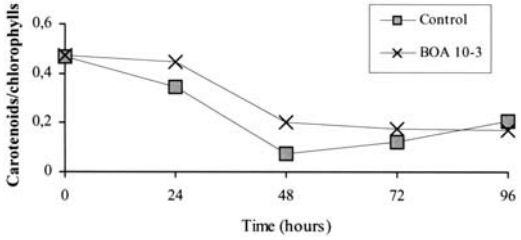

Figure 10.
Carotenoids to chlorophylls ratio for control and BOA 10^{-3} M cultures after 96 h of exposure.

By other hand, it is well known that photosynthesis is closely related to CO_2 balance and that yield increases relate to a decrease of CO_2 in the water. In addition, the CO_2 concentration in water is in equilibrium with the CO_2^{3-} concentration and thus correlated to the pH-value (Weichart, 1985). In consequence, low CO_2 concentrations imply a more alkaline pH-value, which in turn indicates stronger photosynthesis (Bester *et al.*, 1995) and probably faster cell division and growth. The pH values are correlated with the growth data from 0 to 96 h verifying this relationship between metabolic activity and pH-values of the media (R^2 = 0.83 - 0.93, depending on the treatment).

Chlorophyll content may give some idea about the mode of action of the allelochemical in the medium (Kirby and Sheahan, 1994). Decrease of chlorophyll content per unit of plant material does not necessarily mean inhibition of growth and *vice versa* (Verdisson *et al.*, 2001). Concentration BOA 10^{-3} M affects the metabolism of *T. suecica* increasing chlorophyll content per cell when the growth of the algae is reduced with respect to the control. Consequently, chlorophyll content and algal growth are two physiological parameters that can complement each other in toxicity tests. Furthermore, total chlorophyll content in the culture decreases drastically in presence of the highest BOA concentration as a result of growth significant reductions. Algae acclimation to the sub-lethal concentrations of a xenobiotic is demonstrated further by the chlorophyll content per cell (Soukupová *et al.*, 1999). Cells grown in sub-lethal concentrations can compensate the partial inhibition of the photosynthetic capacity by means of an increased photosynthetic antenna that can drive more excitation to the remaining functional reaction centers of PSII. This acclimation process lowers the sensitivity of the algal growth to the sub-lethal concentrations of the applied allelochemical (Soukupová *et al.*, 1999). A similar process was showed by Koening (1990) using DCMU. In addition, it is assumed that chlorophyll synthesis is a major objective in photoauthotrophically grown cultures in absence of cell division (Hagen *et al.*, 2001).

In samples from suspensions exposed to BOA 10^{-3} M where cell division rate was severely diminished during the last three days there was an increase of the chlorophyll *a* to chlorophyll *b* ratio (Fig. 9). The former observation support the fact than some stress situations induce an increase in chlorophyll a (Hagen *et al.*, 2001).

In agreement with Masojídek *et al.* (2000), stop of active cell division in our experimental design was also related to an accumulation of carotenoids per cell. Carotenoids started to accumulate significantly between 48 and 72 h after the exposure to 10^{-3} M BOA. Probably, this process is related with a decrease of the photosynthetic activity (Masojídek *et al.*, 2000). Similar results have been obtained in microalgae submitted to nitrate depletion (Hagen *et al.*, 2001). In those experiments data were not normalized either per cell or chlorophyll and, as a result, comparison among different studies is difficult.

Pigment content revealed a lower carotenoid to chlorophyll ratio in the control culture at 24 and 48 h compared with the most stressed culture (Fig. 10), but the tendency matches the values after 72 h of exposition. Probably, there was an acclimation process (Soukupová *et al.*, 1999).

In our experimental conditions, assessing toxicity of 2(3H)-benzoxazolinone (BOA) on *Tetraselmis suecica* algae widely used in ecotoxicological studies, the allelochemical inhibited algal growth at highest tested concentration. A better estimation of the phytotoxic potential of this allelochemical may be obtained from experiments in which the concentrations of BOA are readjusted daily. We must not belittle that lesser concentrations of BOA should impair the establishment of the phytoplankton by mean of additive or synergistic sub-lethal effects with other compounds that can increase their global toxicity. The possibility that BOA interferes with other photosynthetic parameters is also being investigated.

Because of the similarity of the *T. suecica* physiology with the most of the physiological traits of highest plants and the response obtained to the addition of BOA in these experiments, we can consider this specie as a suitable organism for the analysis of allelopathic activity of natural compounds.

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