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Effects of Nutrients and Salinity on the Algal Assay Using *Pseudokirchneriella subcapitata* (Korshikov) Hindak

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Algal assays are short-term, chronic, sublethal tests conducted with several generations of organisms. Microalgae are used as bioindicators for ecotoxicological hazard evaluation of chemicals (Gaggi et al. 1995; Peterson et al. 1994; Sabater and Carrasco 1997) and for estimating the toxicity of effluents and receiving waters (ASTM 1986; Joy 1990; Galassi et al. 1993; EPA 1994). In algal toxicity test on effluents and receiving waters the indigenous organisms are eliminated by sterile filtration; to eliminate false negative results due to low nutrient concentrations, nutrients are added into both the water sample and the dilution/control water. Microalgae (inocula) are placed in the test chambers containing the sample and the control. Incubation is for 96 hours under standard conditions. Inhibition or stimulation of growth in the sample in respect of the control are the index of either toxic elements or available nutrients.

Complex wastes often contain both growth stimulants and inhibitors. Walsh and Alexander (1980) stated that bioactivity of a complex waste is probably related to interactions among the components, with no substance having a dominant effect. Walsh and Alexander reported that a liquid industrial waste may affect algal growth in any of the three ways: stimulation, inhibition, stimulation at low concentration but inhibition at higher concentrations. There is probably a balance between inhibitory and stimulating factors put into receiving waters by outfalls (Walsh and Merril 1984). When a balance between inhibitory and stimulating factors exists, the toxic effect should be masked.

Nyholm and Kallqvist (1989) stated that algal growth rates are related to intracellular nutrient concentrations, not to extracellular concentrations. Since the growth rate is not influenced by the external nutrient concentrations, complex samples (such as effluent samples) containing nutrients can be tested without interferences from the nutrients added to the sample, if the test duration is restricted to the initial period in which exponential growth prevails in the controls (and in which nutrients are in excess). Nyholm and Kallqvist disagree with the testing philosophy that interprets the increased final yield due to added nutrients in toxicity studies on complex wastes as a stimulatory growth response. They hold the position that toxic effect should not be masked or obscured in a toxicity test as a result of an interaction with nutrients.

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Effluents, receiving waters and irrigation drain waters (Ingersoll et al. 1992) are often saline and contain toxic substances. Salinity can affect freshwater algal growth in toxicity tests, thus a distinction between the toxic effects of salinity and contaminants must be made. The purposes of this work were:

- to evaluate if increasing nutrient concentrations can mask the toxic effect on algal growth;
- to evaluate the effect of salinity on algal growth and to determine the highest salinity value at which no adverse effect is observed.

MATERIALS AND METHODS

The green alga *Pseudokirchneriella subcapitata* (*Selenastrum capricornutum*) was used as test organism. The stock culture was maintained according to EPA (1994) in 250 mL borosilicate Erlenmeyer flasks containing 50 mL culture medium at 24±2°C, under light (16 hrs) and dark (8 hrs) cycle with "Cool-White" fluorescent lamps providing 86 ± 8.6 $\mu E/m^2/s$; the flasks were shaken by hand once a day. Two mL of stock culture were transferred weekly into 100 mL of new culture medium to maintain a continuous supply of "healthy" cells for the tests. The stock nutrient solutions and the nutrient medium were prepared according to EPA (1994). The dilution water was of sufficient quality (ASTM Type I water). All the tests were conducted without EDTA in the culture media.

A series of solutions with increasing concentrations of both phosphorus and nitrogen, was prepared; the algal medium reported in EPA (1994), without phosphorus and nitrogen, was used. The phosphorus enrichment was obtained by adding K_2HPO_4; the nitrogen enrichment was obtained by adding $NaNO_3$. The nutrient concentrations are reported in table 1; the N/P ratio was 22.6, the pH was 7.5±0.1. Each P-N concentration was then identified by the equivalent EPA's culture medium percent concentration (i.e. adding 0.186 mg/L P and 4.2 mg/L N we obtain 100% EPA's culture medium). This series of 150 mL nutrient concentrations was used as control.

Table 1. Nutrient fortification concentrations.

	Nutrient concentration			
	C50%	C100%	C200%	C300%
P (mg/L)	0.093	0.186	0.372	0.558
N (mg/L)	2.1	4.2	8.4	12.6

A second series of nutrient concentrations was set up to test the effects of 0.1 mg/L chromium concentration. An aliquot of 0.43 mL of $K_2Cr_2O_7$ stock solution (35 mg/L for Cr) was added to 150 mL of each nutrient solution. Potassium dichromate can be used as a reference substance as reported in OECD (1984) and ISO (1989). Three replicates were set up for each treatment and control. Twenty-four batch cultures were prepared in flasks of 250 mL; each flask contained 50 mL of test solution. Inoculum was prepared according to EPA (1994); 0.5 mL of 1,000 x 10^3 cells/mL algal culture were then inoculated into each flask. The

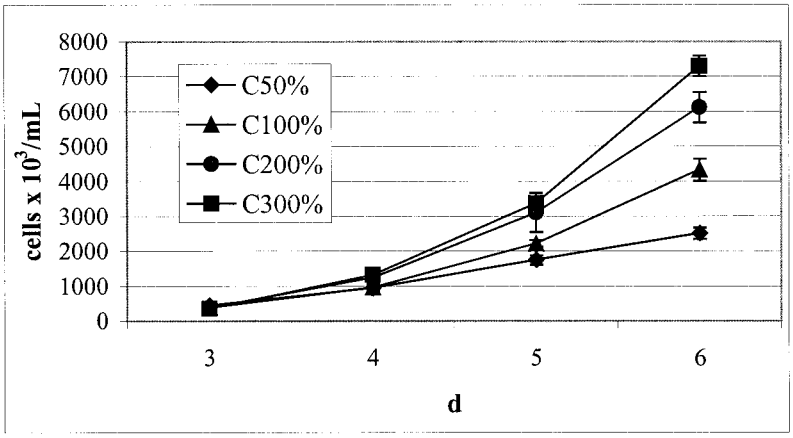
initial cell density was approximately 10,000 cells/mL (± 10%). The test flasks were incubated under the continuous illumination, the growth irradiance and the temperature levels which are described above. Five-mL aliquots of culture were withdrawn from each flask in the 3rd, 4th, 5th and 6th day respectively. These aliquots were used to determine the algal growth by algal density measures and chlorophyll a *in vivo* concentration measures. Algal density (cells x 10^3/mL) was measured by electronic particle counter (Coulter Counter, Model Z1) and counted using a 100-μm diameter aperture. Chlorophyll a *in vivo* concentration (μg/L) was measured by fluorometer Turner Model 10-AV (emission at 680 nm wavelength after excitation at 436 nm). Calibration between fluorescence intensity and chlorophyll a *in vivo* concentration was made by using the chlorophyll extraction procedure. Fluorescence yield is a function of chlorophyll concentration and chloroplast shape (Walsh and Merril 1984).

A concentrations series was prepared adding sodium chloride to EPA medium to evaluate the toxicity of salinity. Ion chloride concentrations ranged from 0.5 g/L to 4.0 g/L. The control solution was prepared using EPA medium without sodium chloride. Three replicates were set up for each concentration and the control, the algal growth was determined after 72 hrs of incubation, the test conditions being the same described above. EPA's acceptability criteria (EPA 1994) were met.

RESULTS AND DISCUSSION

After 3, 4, 5 and 6 days of incubation the mean growth for each treatment and for the relative control with the same nutrient concentration was determined. Figure 1 shows the time course of the changes in algal growth and chlorophyll content with varying concentration of nutrients in the controls. Figure 2 shows the inhibition % of algal growth for each treatment in respect of the relative control with the same nutrient concentration. The inhibition of growth, determined for the treatment T100% by cell count at the 3rd exposure day, compared to the relative control (C100%), was not significant with t Student test ($p>0.05$); all the other growth reductions were significant. In algal assay the chromium toxicity does not change with respect to the increase of nutrient concentration when the latter is the same in the treatment (sample) and in the control.

At the end of the 3rd, 4th, 5th and 6th days of incubation the mean values of the algal growth for C100% (control) and T50, T100, T200, T300% (treatments) were compared using the Dunnett's Procedure (EPA 1994). The control C100% was used as reference because this nutrient concentration is utilized as control in effluent and receiving water algal bioassay (EPA 1994); T50, T100, T200 and T300% treatments could represent effluent or receiving water samples with constant toxicity and increasing nutrient concentrations. In figure 3 the apparent inhibitions % of algal growth measured as algal density and chlorophyll a *in vivo* content are reported. When the nutrient concentration in the treatments was either the same or lower than the control (C100%) the growth inhibition % was not affected in the time course. When the nutrient concentration in the treatments was higher than the control (C 100%) the growth inhibition % was affected.



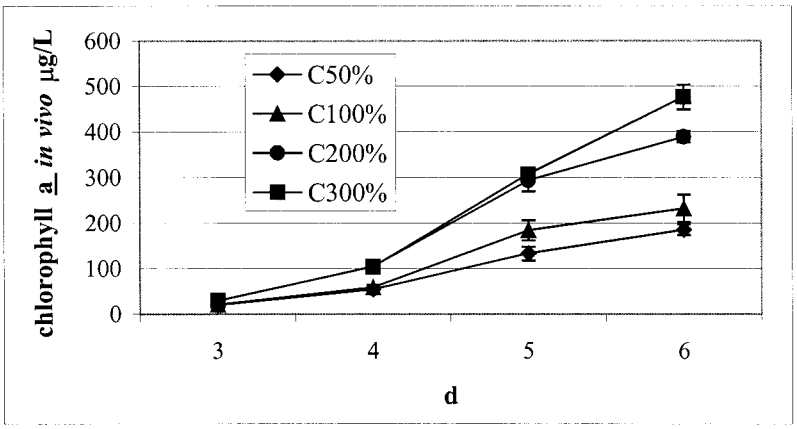


Figure 1. Algal growth in the controls measured by cell density and chlorophyll a *in vivo* content. Means are plotted with error bars indicating standard deviation.

The highest nutrient concentration, corresponding to 300%, always masked the toxic effect when the cell density determination system was used; also the 200% nutrient concentration masked the toxic effect after 6 days of exposure. Both 200 and 300% nutrient concentrations always masked the toxic effect when the chlorophyll a *in vivo* determination system was used. Both high concentration of nutrients in the treatments in respect of the control and a long exposure time supported the masking effect on the toxicity. The algal growth measured at the 4th day corresponds to the termination of the algal test, according to EPA (1994) and ASTM (1986). After the fourth day of exposure, significant variations among the controls (C50%, C100%, C200%, C300%) were found by using both determination systems (ANOVA, $p<0.05$). The algal growth was positively correlated with the nutrient concentration using both determination systems

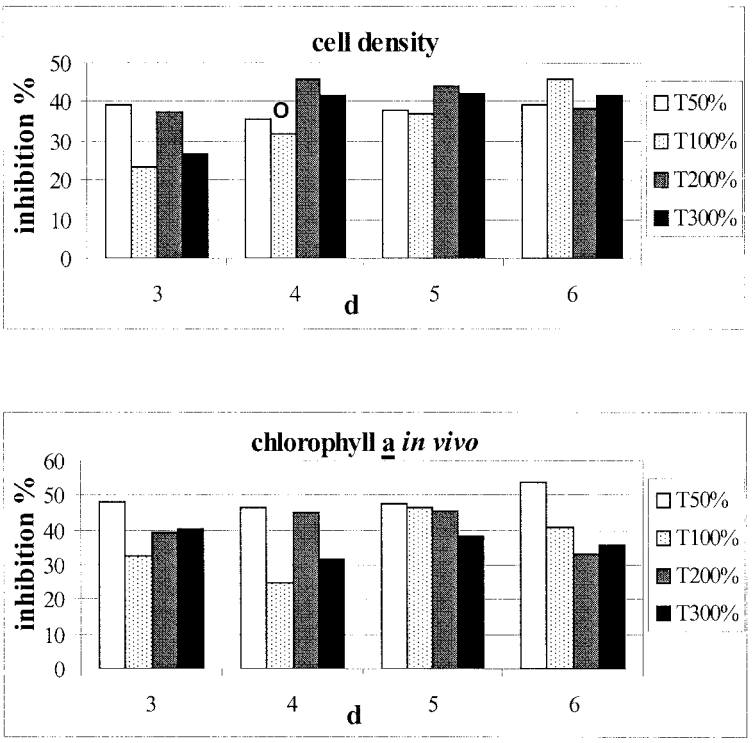


Figure 2. Inhibition % of algal growth as cell density and chlorophyll a *in vivo* content for each treatment in respect of the relative control with the same nutrient concentration. Each treatment contains 0.1 mg/L of chromium concentration. All but one measurements show significant differences. (o) Not significant differences, t student test, $p>0.05$.

(regression equation for the measures of cell density: $y = 1.6335x + 849.58$, $R^2 = 0.655$; regression equation for the measures of chlorophyll a *in vivo*: $y = 0.23x + 43.21$, $R^2 = 0.779$). On the contrary, after the third day of exposure no significant variations among the controls were found by using both determination systems (ANOVA, $p > 0.05$).

After the third day of incubation, the toxicity of salinity was determined (Figure 4). $EC50^{72h}$ values were obtained by using probit analysis; NOEC values were obtained by using the Dunnett's procedure (EPA 1994). The test was replicated 20 times. The $EC50^{72h}$ mean value (± confidence interval) was 1.9 (±0.1) g/L Cl^- and the NOEC mean value was 1.2 (±0.2) for the measures of cell density. The $EC50^{72h}$ mean value was 2.3 (±0.2) g/L Cl^- and the NOEC mean value was 1.5 (±0.3) for the measures of chlorophyll a *in vivo*. T student test was used to evaluate statistically the difference between EC_{50}^{72h} values obtained with particle

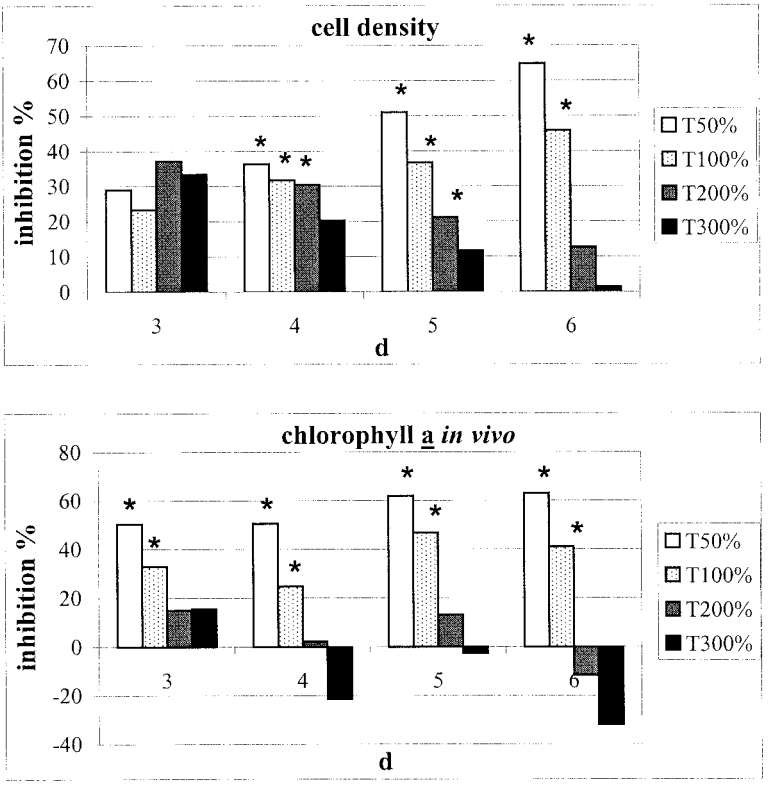


Figure 3. Inhibition % of algal growth as cell density and chlorophyll a *in vivo* content for each treatment in respect of control C100%. (*)Significant differences, Dunnett test, $p<0.05$.

counter and fluorometer measures. The results showed that there was no statistically significant difference ($p > 0.05$). No statically significant difference ($p>0.05$) was found for NOEC values, either. Utz and Bohrer (2001) measured the ion chloride toxicity with *Daphnia similis* to evaluate the hazard of salinity of the drilling fluid. The acute toxicity measured as $EC50^{48h}$ with *D. similis* is of the same order of magnitude as regards the toxicity measured with *P. subcapitata*, reported as EC_{50}^{72h}. As a general rule, EPA recommends that freshwater organisms should be used when the water sample salinity is less than 1 g/L, whereas marine organisms should be used when the water sample salinity equals or exceeds 1 g/L (EPA 1991). According to EPA, NOEC values obtained in the present work confirm the use of *P. subcapitata* only for freshwater samples when the salinity is less than 1 g/L.

In conclusion, after the fourth day of exposure the algal growth was influenced by nutrient concentration; high values of the latter in samples in respect to the control masked the toxic effect of chromium. Both the use of the cell density

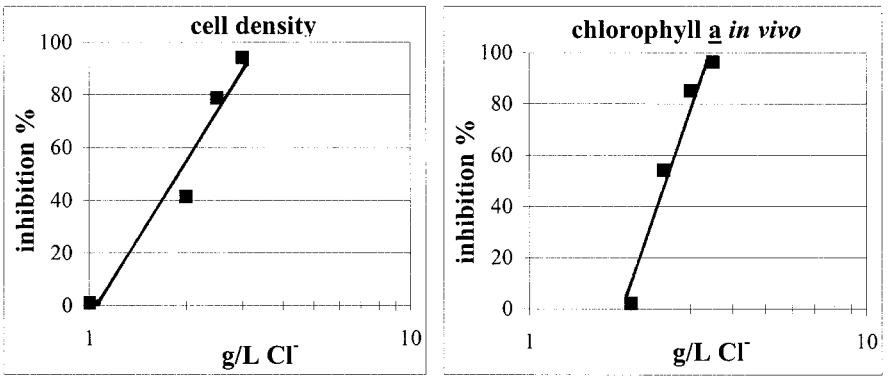


Figure 4. Toxicity of NaCl; inhibition % of algal growth as cell density and chlorophyll a *in vivo* content.

determination system and a short exposure times allow the reduction of the masking effect.

Salinity is a parameter that must be checked in the sample prior to *P. subcapitata* toxicity test initiation. When its concentration is higher than 1 g/l the algal growth is inhibited and it could be necessary to distinguish between the toxic effect of salinity and other contaminants by using saltwater organisms.

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