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Oxygen Production Rate as a Test for Determining Toxicity of Copper to *Rhodomonas salina* Hill and Wehterbee (Cryptophyceae)

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Unicellular algae are primary producers widely used for assessing the impact of toxics in aquatic environments (Bitton & Dutka, 1986). Most of the usual tests involving microalgae have been based on population growth (OECD, 1984; USEPA, 1985), respiratory processes (Pérez-García et al., 1993), or ^{14}C assimilation (Kusk & Nyholm, 1991). European Community, in modification L 259/10 of Directive 67/548/CEE (related to the approach of legal dispositions related to packaging and labelling of dangerous substances) includes an ecotoxicological assay of the test substance on an undeterminated algal species (CEE, 1979). Most accepted tests take three or four days (OECD, 1984). Thus, a faster, sensitive toxicity test would be useful.

In this work, the marine microalga *Rhodomonas salina* Hill & Wehterbee (Cryptophyceae) is proposed as a suitable species to be used in a fast (24 hours) test based on the measurement of oxygen production. In some conditions, Cryptophyceae dominate the phytoplanktonic polulations in open ocean. Aditionally, species from this Class are represented during all year (Klaveness, 1988) Results of this test was compared with the classical OECD microalgal test and Microtox test for the same toxicant.

MATERIALS AND METHODS:

Rhodomonas salina Hill and Wetherbee (Cryptophyceae) was selected because of the high sensitivity of this species to heavy metals (Moreno-Garrido, 1997). The strain used was obtained from the Marine Microalgae Culture Collection at the Instituto de Ciencias Marinas de Andalucía (CSIC) (Lubián and Yúfera, 1989). Cells were cultured for one month before starting the experiments in the same medium as that used in toxicity tests . Cells used were exponentially growing when selected for testing. Microalgae were cultured in artificial sea water from Sigma Chemicals. After adding salts, pH was adjusted to 8.6 using small volumes of 0.1M NaOH and 0.1M HCl , then filtered through a 0.45 µm filter (Millipore) and sterylized during 15 minutes in an autoclave. After sterylization, pH remained at 8.0 ± 0.2. Water more than five days old was discarded. Nutrient solution was a

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modification of Guillard's f/2 (Guillard and Ryther, 1962), without EDTA because this chelator reduces the toxicity of heavy metals (Moreno-Garrido et al., 1997). Exposure to copper occurred at 24°C under continuous white light (300 $\mu E m^{-2}s^{-1}$) in a Koxka culture chamber, in 100 mL sterile flasks, following OECD guideline (OECD, 1984).

All experiences were carried out in triplicate. Copper doses used in photosynthesis tests were in the same range as those used in OECD (1984) growth inhibition tests. Total oxygen production rate was measured using an Clark-type electrode (Hansatech, Kings Lynn, UK) equipped with an 1 mL measuring chamber provided of an outer jacket for thermostat-controlled water. Light intensity inside the chamber was 1050 $\mu E\ m^{-2s-1}$. After few seconds (necessary for adaptation of microalgae to the measuring chamber) total oxygen production rate was calculated from the slope of the dissolved oxygen level curve (Lubián and Montero, 1998). Inhibition average was calculated by comparison of the oxygen production rate of the cultures exposed to the different copper doses with the oxygen production rate of the blank cultures. The data of averages of oxygen production rate versus doses of copper (this latter in logarithmic scale) were fitted to a straight line, and from the equation of this line $EC50\%_{tot}24h$ was calculated. In the same way as in the OECD guideline (OECD, 1984), at least 50% of inhibition must be reached within the range of experimental doses to validate the test. A measuring cellular concentration equivalent to 0.42 µg Chlorophyll a mL^{-1} was chosen because smaller amounts of cells would not produce oxygen enough to be detected by the oxygen electrode (Huertas, 1995). But since sensitivity of a population of microalgae greatly depends on cellular density (Moreno-Garrido, 1997), that is, the lower cellular density, the higher sensitivity, cells were exposed to copper at a cellular concentration six times lower than the measuring concentration ($77x10^3$ cells mL^{-1}), in order to use cellular densities similar to those recommended in OECD guidelines (OECD, 1984). Just before measuring (24 hours after inoculation), 6 mL of culture were centrifugated during 3 minutes at 13000 rpm (11600 g) in a MSE desktop microcentrifuge. Pellets were transferred to a final volume of 1 mL clean artificial sea water with 2 mM HCO_3Na, so as to improve photosynthesis. This is the normal level of bicarbonate in ocean waters (Skirrow, 1975).

Microtox test, based on fluorescence of *Vibrio fisherii*, was developed following instructions of the test (Isemberg, 1993). Toxicity to copper was measured at 5 and 15 min.

Copper was added as copper (II) sulphate pentahydrate, reagent grade, provided by Merck. Copper concentration in the medium was certified by measuring with Atomic Absorption Spectrophotometry (flame technique), after acidification with nitric acid. All glassware was cleaned with diluted nitric acid and rinsed several times with Milli-Q ultrapure water.

RESULTS AND DISCUSSION:

After a previous experience developed in order to find the range of toxicity of copper to *R. salina* (this results are shown in Figure 1), EC50%72h for biomass was calculated following OECD guidelines (OECD, 1984), comparing the area under the growth curves. Microalgal population growth curves for different copper doses are shown in Figure 2. Doses in logarithmic scale versus inhibition average, along with the fitted stright line for calculating EC50%72h, are shown in Figure 3. EC50%72h for copper resulted to be 0.03 mg L^{-1} Cu. The correlation coefficient of fitted straight line to a semilogarithmic plot of growth inhibition average versus toxic metal concentration data was 0.988.

Microtox test was applied for copper, at 5 and 15 minutes. Inhibition of 50% of fluorescence for *Vibrio fisherii* was reached at 0.15 mg L^{-1} Cu at 5 minutes and 0.12 mg L^{-1} Cu at 15 minutes. In them both cases, 95% confidence range was within ± 0.007 mg L^{-1}.

Measurements of total oxygen production rate at 24°C were carried out for selected copper concentrations after 24h of exposure. Results are shown in Figure 4. The average of photosynthesis inhibition can be calculated by comparing the total oxygen production rate of microalgae exposed to different copper doses with the total oxygen production rate of control microalgae (assuming 0% ofinhibition for this latter). When plotting in semilogaritmic scale, inibition data fit to a straight line (Figure 5). From equation of this line, EC50%24h for photosynthesis (EC50%p24h) can be calculated. The value of EC50%p24h (0.03 mg L^{-1}) obtained here is identical to the EC50%72h for biomass obtained following OECD, but results can be obtained in 24 hours with our test. At least for copper, tests with this microalgal species are more sentitive than microtox test.

Lüderitz and Nicklisch (1989) found growth inhibition values of 0.064 and 0.08 mg L^{-1} Cu for *Aphanizomenon gracile* and *Oscillatoria redekei,* respectively at similar pH values to those used in the present work (pH 8.2). Abalde *et al.* (1995) did not find toxic effects in populations of *Dunaliella tertiolecta* with higher copper doses (8 mg L^{-1}), but these data must be carefully revised: culture medium used in these experiments was described in Fábregas *et al.* (1986) and EDTA concentration in that medium is 8.6 mg L^{-1}. This could affect toxic capacity of copper by chelating processes. In addition to this, iron is added as citrate salt in this medium, and citrate can act as chelator too.

Most of toxicity or biostimulation tests carried out with marine phytoplankton have been developed on green microalgae (i.e. *Dunaliella* sp., *Chlorella* sp.), or diatoms (i.e. *Talassiosira pseudonana, Skeletonema costatum, Phaeoductylum tricornutum, Nitzschia punctata*) (Clesceri et al., 1989; Rand, 1995). There are some data about sensitivity of dinoflagellates: inhibition of mobility in *Gonyaulax tamarensis* occurs at copper concentrations in the medium of 1 µg L^{-1} (Anderson

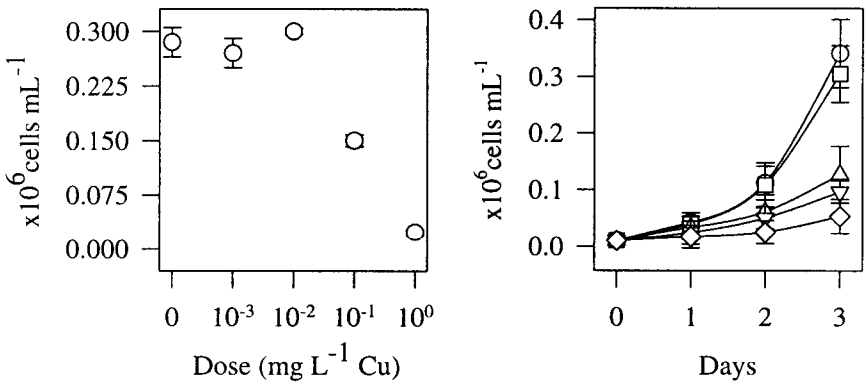

Figure 1 (left). Cellular densities for *Rhodomonas salina* after 72 hours growing when exposed to different doses of Cu. Error bars mean standard deviation between replicates.

Figure 2 (right): Grouth curves for populations of *Rhodomonas salina* during 72 hours exposed to different doses of Cu (mg L^{-1}). ○= control;□ = 0.01;△ = 0.04;▽ =0.05;◇=0.07. Error bars mean standard deviation between replicates.

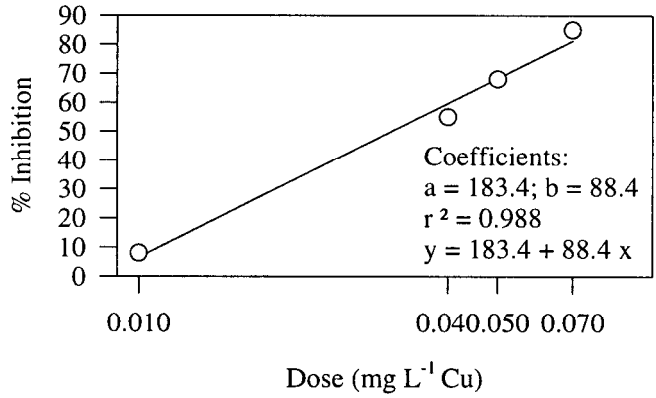

Figure 3: Regression line over plotted values of growth inhibition for *Rhodomonas salina* in semilogarithmic scale. Equation of the fitted line is represented too.

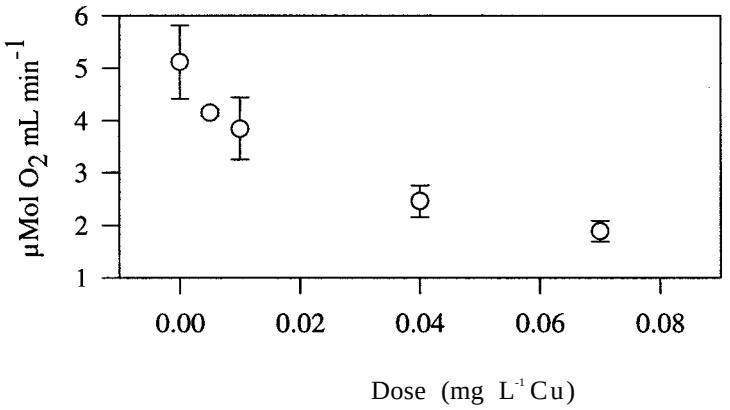

Figure 4: Total oxygen production rate (measured as µmol O_2 mL^{-1} min^{-1}) after 24 hours exposure of populations of *Rhodomonas salina* to selected Cu doses. Error bars means standard deviations between replicates.

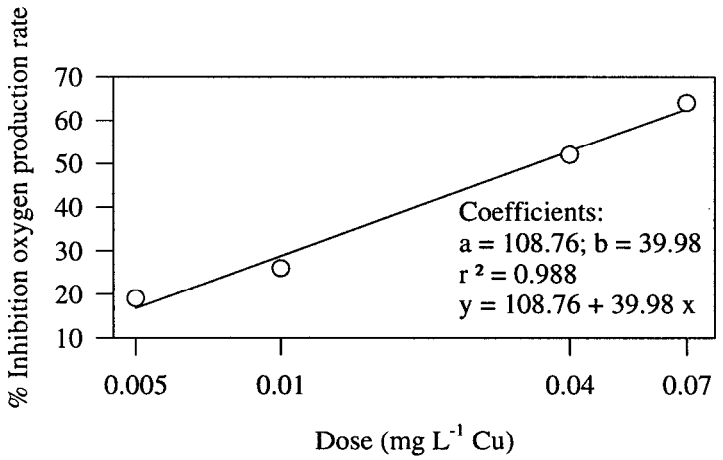

Figure 5: Regression line over plotted values of total oxygen production rate inhibition for *Rhodomonas salina* in semilogarithmic scale. Equation of the fitted line is represented too.

and Morel, 1978). No data have been found in the literature about toxicity tests involving Cryptophyceae. In freshwater tests, Chlorophyceae and Prasynophyceae seems to be more resistant than other microalgal classes, but all recommended microalgal species for testing chemicals following OECD guidelines belongs to Chlorophyceae (OECD, 1984). Cedeno-Maldonado and Swader (1974) reported inhibition of photosynthesis and respiration in *Chlorella* sp, after two minutes of exposure, but only when exposed to concentrations as high as 63.54 mg L^{-1} Cu. These high copper levels could not be significant, because toxic effects in environment would occur at quite lower concentrations.

Toxicity tests have been developed in order to know the potential damage to biota caused by single toxicants or effluents. Selection of sensitive organisms to toxicants to be used in toxicity tests would ensure a better protection of biodiversity in aquatic environments.

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