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The Effect of Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} on the Growth Rate of Marine Diatom *Phaeodactylum tricornutum* Bohlin: Microplate Growth Inhibition Test

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Algae are predominantly aquatic organisms that must be able to discriminate between essential and non-essential heavy metal ions (Perales-Vela et al. 2006). Many of the trace metals are essential but toxic at elevated concentrations. Cobalt (Co^{2+}), although an essential element (Lustigman et al. 1995), seems to have a direct effect on P680 (El-Sheekh et al. 2003). According to El-Sheekh et al. (2003) higher Co^{2+} concentrations had an inhibitory effect on O_2 uptake by the two algal species *Monoraphidium minutum* and *Nitzchia perminuta*. Zinc (Zn^{2+}) is also an essential metal and at elevated concentrations can be toxic and cause algal cell death. Hirata et al. (2001) established that in marine green algae *Dunaliella tertiolecta* glutathione and phytochelatins can be involved in Zn detoxification. Mercury (Hg^{2+}), a toxic but non-essential element, is a metal with redox capacity that can enhance the pro-oxidant status by reducing the antioxidant glutathion pool (Okamoto et al. 2001). Cadmium (Cd^{2+}) has no known biological functions and is a highly toxic metal to aquatic organisms. Some previous studies demonstrated that Cd can be a substitute of Zn in marine phytoplankton (Price and Morel 1990). Nickel is one of the toxic metals found in industrial wastewaters (Lustigman et al. 1995). Nickel caused reduction in dry weight and at the same time highly stimulated biosynthesis of sulfur containing amino acids in *N. perminuta* probably due to some chelating mechanisms against heavy metal toxicity (Osman et al. 2004).

As pointed out in the paper of Pavlić et al. (2006) microplate algal assays for freshwater algae offer many advantages over standard Erlenmeyer flask tests. A similar procedure was developed for marine algae. Lukavský and Simmer (2001) miniaturized the ISO 10253 bioassay with the marine diatom *Phaeodactylum tricornutum* by using immunological plates with wells of 0.25 and 2.5 ml as cultivation vessels. Diatoms occupy a variety of habitats and are often the most abundant photosynthetic organisms in marine waters. *P. tricornutum* is one of the most used algal species in the marine bioassays because of its easy cultivation, it is also a prospective alga in aquaculture as food for, e.g., crustacean *Artemia* (Kvíderová and Lukavský 2003). Therefore, in this study we have investigated the influence of different heavy metals (Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}) on the growth rate of marine phytoplankton diatom *P. tricornutum* Bohlin (Bacillariophyceae) by miniaturized bioassay method.

Materials and Methods

The toxicity tests were carried out with the unicellular marine diatom *P. tricornutum* Bohlin 1897 strain CCAP 1052/1A obtained by Culture Collection of Algae and Protozoa, UK. The alga was cultivated at the Department of Biology, J. J. Strossmayer University in Osijek in the f/2 medium at 20°C under continuous white light exposed to irradiance by PAR 120 μmol $m^{-2}s^{-1}$ measured with flat sensor. The basic design of toxicity tests followed the International Standard ISO 10253 marine algal growth inhibition test (ISO 1995) and were modified according to Lukavský (1992) and Lukavský and Simmer (2001). To determine the toxicity of metals (Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}), on the growth rate of marine phytoplankton diatom *P. tricornutum* Bohlin, nickel sulphate ($NiSO_4$ $6H_2O$), cobalt chloride ($CoCl_2{\cdot}6H_2O$), zinc sulphate

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($ZnSO_4 \cdot 7H_2O$), cadmium nitrate ($Cd(NO_3)_2 \cdot 4H_2O$), mercury chloride ($HgCl_2$) and mercury sulfate ($HgSO_4$) of analytical grade (99%, Kemika, Zagreb) and cobalt nitrate ($Co(NO_3)_2 \cdot 6H_2O$) of analytical grade (99%, Fluka) were used. A range of nominal concentration of test medium was prepared (from 0.01 to 5 mg L^{-1} for Hg, 0.02 to 10 mg L^{-1} for Co as $CoCl_2$, 0.16 to 40 mg L^{-1} for Cd, 0.04 to 80 mg L^{-1} for Ni and 0.08 to 80 mg L^{-1} for Zn and Co as $Co(NO_3)_2$). Toxicity tests were carried out in polystyrene 96-well microplates (TPP, Switzerland) with 9 × 13 cm flat bottom wells of 300 µL. Peripheral wells were filled with distilled water to reduce evaporation; six replicates per test solution were located in columns, from the lowest to the highest concentration. Test solution volume was 200 µL. The laboratory control was *f*/2 medium. The uncovered plates with test solutions were exposed for 3 h to UV light for sterilization. The final algal density of *P. tricornutum* was 10^4 cells/mL and was determined using a Bürker–Türk counting chamber (Karl Hecht KG, Sondheim, Germany) under a light microscope (Axiovert 25, Carl Zeiss, Inc., Göttingen, Germany). These inoculum's solutions were obtained from a preculture, which was incubated under test conditions and used when cells were exponentially growing (3–5 days old culture). After the algal inoculation the microplates were closed with lids and exposed to cultivation conditions in Phytotron (Voetsch). The growth of *P. tricornutum* was determined by measuring the optical density (OD) at 750 nm at 0, 24, 48, 72, 96, 168 and 336 h, using an automated microplate reader (Multiskan MS, Labsystem, Finland) controlled by GENESIS II software (Windows™ Based Microplate Software). The growth rate (μ) was calculated from the absorbance increase per day assuming the logarithmic increase in biomass according to $\mu = \ln(A_n/A_0)/(t_n - t_0)$ where A_0 and A_n were absorbances at 750 nm at the start of the test (t_0) and after n days (t_n), respectively. The toxic response was expressed as growth inhibition (I%) calculated as the relative reduction in growth rate (μ) caused by the test substance: $I = 1 - (\mu_{inhibited}/\mu_{control})$. The IC50s were determined from the polynomial curve fitted to the data by the least squares method. The data were located on the concentration – inhibition curve.

Results and Discussion

The effect of metal ions (Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+}) on the growth rate (μ) of *P. tricornutum* was investigated by growing cells in culture media to which metals were added. According to Toress et al. (1997) growth is a good indicator of the toxic action of metals in microalgae and reflects the metabolism of the cell. So, because the specific growth rate is considered a reproducible ecologically relevant response and not strongly test system specific, it was selected as an appropriate test endpoint. Growth rate of *P. tricornutum* as a function of tested concentrations at the exponential and at stationary phases of growth are shown in Figs. 1 and 2. By comparing these toxicity curves the differences in slopes could be observed. Zn, Cd, Co and Ni have a gradual "slow" curve, while Hg has a "sharp" one (Figs. 1, 2) thus indicating high toxicity of Hg.

Compared with the control culture (no metal added, growth rate (μ) of 0.114 day^{-1} after 72 h, and μ = 0.072 day^{-1} after 336 h) the percent of growth inhibition was calculated for each test concentration. The inhibition percent values of tested metals at the exponential phase (after 72 h) and at stationary phase of growth (after 336 h) are shown in Figs. 3 and 4. A significant stimulation of growth rate (>10%) in the exponential phase of growth was found for Cd^{2+} (0.16–0.31 mg L^{-1}) and Co^{2+} as $Co(NO_3)_2$ from 0.08 to 1.25 mg L^{-1} and as $CoCl_2$ from 0.02 to 0.16 mg L^{-1} (Figs. 1, 3). After 72 h of experiment duration the highest concentration that caused significant inhibition of *P. tricornutum* growth rate was the cadmium concentration of 5 mg L^{-1}, therefore including *P. tricornutum* in the group of the most tolerant organisms to cadmium. Toress et al. (1997) also found that *P. tricornutum* is one of the most tolerant algae to cadmium toxicity with the IC50-96 h value of 22.39 mg L^{-1}. According to Scarano and Morelli (2003), *P. tricornutum* is able to incorporate Cd induced sulfide ions in Cd-phytochelatin complexes, thus forming nanometer sized phytochelatin-coated CdS nanocrystalites. The formation of phytochelatin-coated CdS nanocrystalites could explain the low sensitivity to Cd of this alga, as compared with other algae (Toress et al. 1997).

Compared with the control *P. tricornutum* cells grown for 336 h at concentrations between 0.08 and 2.5 mg L^{-1} of Co^{2+} as $Co(NO_3)_2$, between 0.08 and 0.31 mg L^{-1} of Zn^{2+}, at concentration of 0.02 mg L^{-1} of Co^{2+} as $CoCl_2$ and of 0.16 mg L^{-1} of Cd^{2+} showed a significant stimulation of growth (Figs. 1, 3). It can be concluded that low concentrations of Cd and Co had stimulatory effect on the growth rate of *P. tricornutum* during both the exponential phase and the stationary phase of growth, while Zn showed stimulation effect only in the stationary phase of growth (after 336 h). Stimulation indicates some function of the metal in the metabolism of the organism. According to Morelli and Scarano (2001), in contrast to Cd, *P. tricornutum* exposed to Zn did not show PC synthesis or glutathione depletion, although Zn accumulated into the cell at levels comparable to those found in Cd. They supposed that a significant fraction of the intracellular Zn was bound to other cellular molecules and components decreasing the efficiency to activate phytochelatin synthetase. Price and Morel (1990) have reported

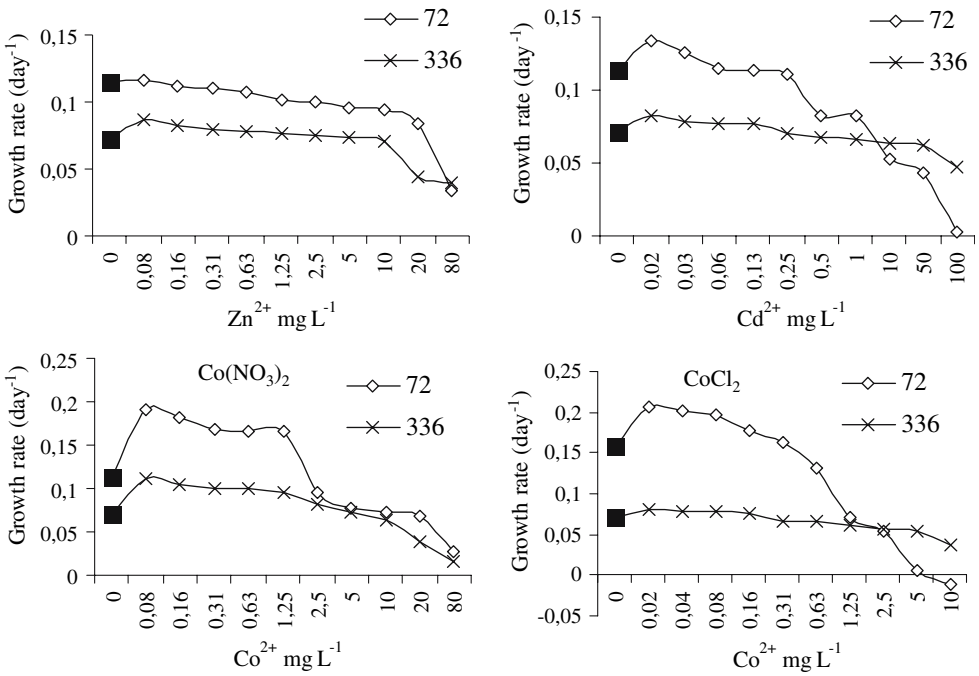

Fig. 1 Growth rate of *P. tricornutum* as a function of tested concentrations of Zn, Cd and Co for 72 and 336 h of exposure duration. *Filled square* control growth rate

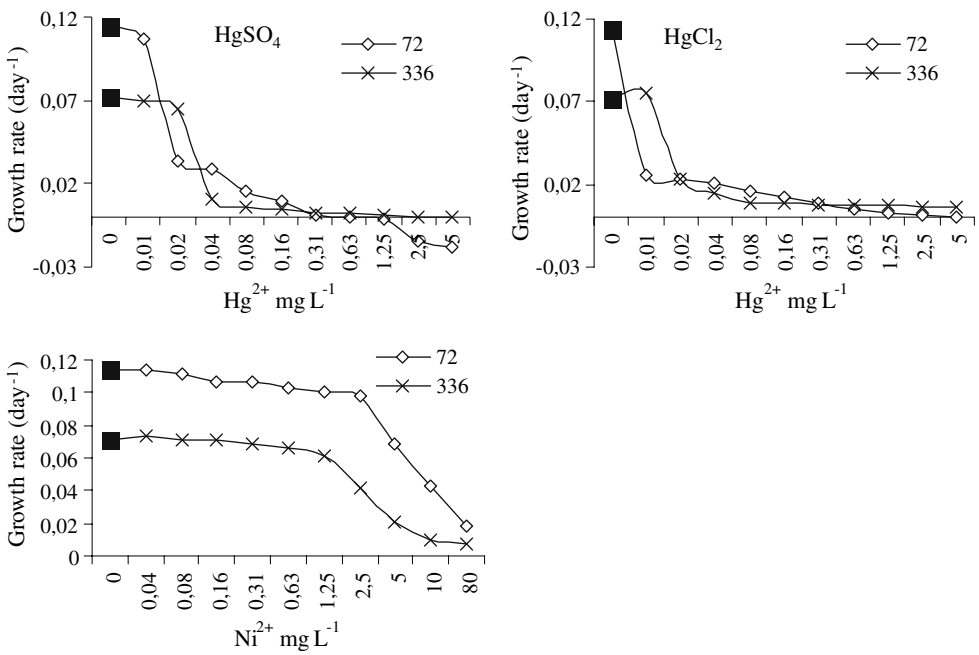

Fig. 2 Growth rate of *P. tricornutum* as a function of tested concentrations of Hg and Ni for 72 and 336 h of exposure duration. *Filled square* control growth rate

that growth stimulation of low cobalt concentrations may be due to cobalt substitution for Zn in some metalloenzymes.

The IC50 values (mg L^{-1}) of different intervals (72, 96, 168 and 336 h) were calculated as shown in Table 1.

The following order of toxicity was achieved: Hg>**Cd**>Ni>Co>Zn in the exponential phase of growth (72 h), and Hg>Ni>Co>Zn>**Cd** after 336 h of experiment duration (in the stationary phase of growth) with mercury as the most toxic metal. Although toxic, the effect of Hg could be attenuated under chronic conditions. Okamoto et al. (2001) demonstrated that under chronic Hg^{2+}, Cd^{2+} and Pb^{2+} treatment *Gonyaulax polyedra* showed reduced light harvesting capacity due to low peridinin levels. Such antioxidant response at the subcellular site could contribute to the overall tolerance of this alga. Since the IC50 values for Hg did not change significantly it seems that *P. tricornutum* develops a resistance to Hg.

Table 1 IC50 (mg L^{-1}) values of different intervals (72, 96, 168 and 336 h) for tested metals (Cd^{2+}, Zn^{2+}, [a]Co^{2+} as $Co(NO_3)_2$, [b]Co^{2+} as $CoCl_2$, [c]Hg^{2+} as $HgSO_4$, [d]Hg^{2+} as $HgCl_2$ and Ni^{2+}) determined from the polynomial curve fitted to the data by the least squares method; *r* is the correlation coefficient, number of concentrations was n = 10

	Cd^{2+}	Zn^{2+}	[a]Co^{2+}	[b]Co^{2+}	[c]Hg^{2+}	[d]Hg^{2+}	Ni^{2+}
IC50-72 h	5.368	41.85	19.57	1.19	0.03	1.16×10^{-5}	7.28
r	0.98	0.96	0.96	0.97	0.85	0.99	0.92
IC50-96 h	15.72	32.89	19.19	5.92	0.04	6.89×10^{-6}	4.59
r	0.98	0.93	0.96	0.92	0.75	0.92	0.93
IC50-168 h	188.68	34.64	24.41	8.97	0.04	0.01	3.58
r	0.95	0.83	0.96	0.95	0.74	0.66	0.92
IC50-336 h	7560.6	140.63	34.29	36.45	0.07	0.06	2.96
r	0.94	0.89	0.96	0.95	0.77	0.67	0.93

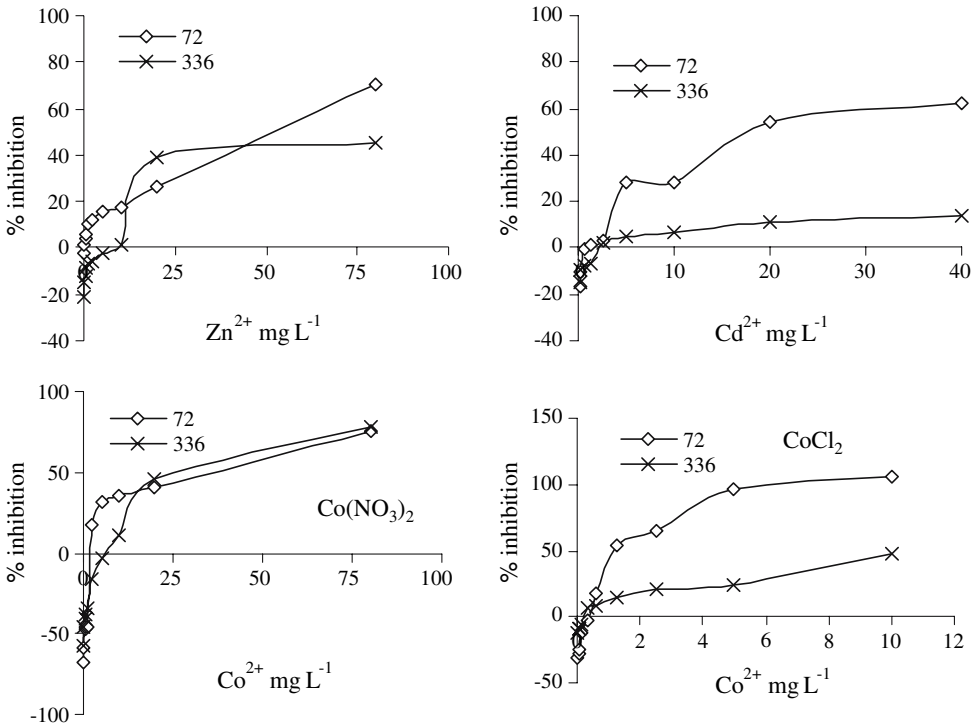

Fig. 3 Percent of inhibition of Zn^{2+}, Cd^{2+} and Co^{2+} on the growth rate of *P. tricornutum* over a 72 h exposure period (exponential phase) and a 336 h exposure period (stationary phase of growth)

Phaeodactylum tricornutum in the exponential phase of growth showed more tolerance to nickel than cadmium. In the stationary phase of growth a high tolerance to cadmium, zinc and cobalt was observed, while nickel showed an increase in toxicity (Table 1). Nickel is a pollutant with a very low binding capacity in many microalgal species. Macfie and Welbourn (2000) found that metals in increasing order of affinity for the cell wall in *Chlamydomonas reinhardtii* were Ni, Co, Cd and Cu, what matches the order of total metal content of the cell with Ni being accumulated in the least amounts. Observing the IC50 values for all tested metals it can be seen that long-term exposure of *P. tricornutum* to Co^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} leads to decreased sensitivity with the exception of Ni^{2+} which shows an opposite effect. *P. tricornutum* is one of the most used algal species in the marine bioassays, and a prospective alga in aquaculture as food for, e.g., crustaceans. Therefore, environmental influence of heavy metal toxicity combined with increased eutrophication can affect phytoplankton community and abundance leading to increased metal uptake and thus directly influencing on trophic transfer of metals along the food chain. So, besides providing screening toxicity profiles this method can be useful in determining the effects of pollutants on cell growth and viability of organisms. Also, establishing the maximum tolerance levels for organisms, this method could be used for evaluating the potential impact of pollution on aquatic system.

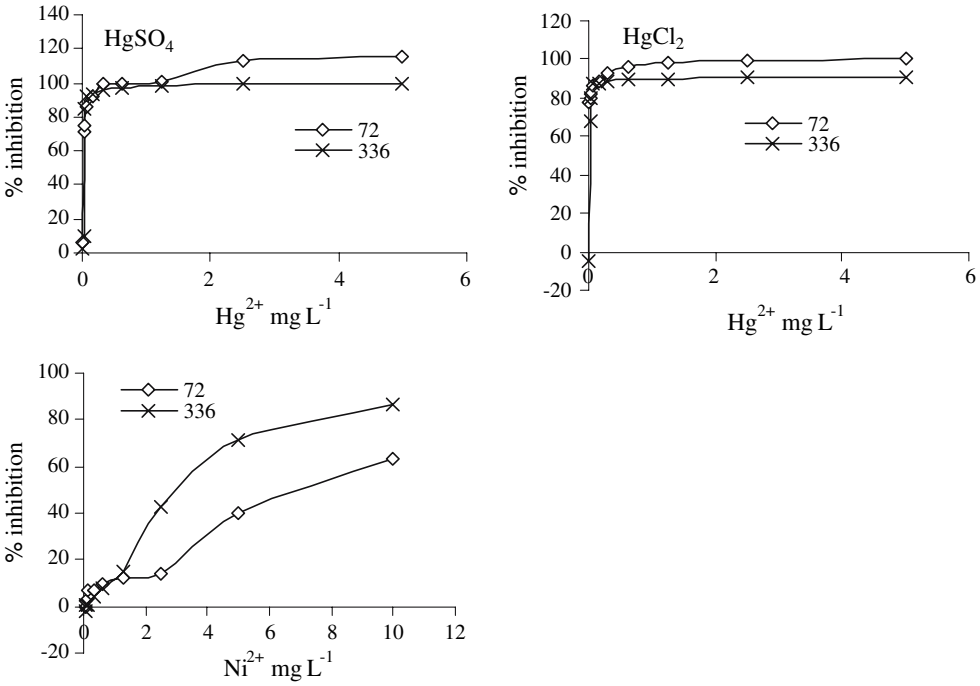

Fig. 4 Percent of inhibition of Hg^{2+} and Ni^{2+} on the growth rate of *P. tricornutum* over a 72 h exposure period (exponential phase) and a 336 h exposure period (stationary phase of growth)

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