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TOXICITY OF LEAD AND CADMIUM TO TROPICAL MARINE PHYTOPLANKTON

Susanne Dal Jensen[1], Suwanna Panutrakul[2] and Niels Nyholm[1]

[1]*Department of Environmental Science and Engineering, Technical University of Denmark.*
[2]*Phuket Marine Biological Center, Thailand.*

ABSTRACT

The toxicity of Pb and Cd to three tropical, marine phytoplankton species isolated from the Andaman Sea off Phuket Thailand were determined. The phytoplankton species included one diatom, *Chaetoceros calcitrans*, one green alga, *Chlorella* sp., and one chrysophyte, *Dunaliella tertiolecta.* The test method was a two day mini scale (10 mL) modified International Standard (ISO) growth inhibition test with natural and artificial seawater. Citric acid was added as a metal chelator instead of the more strongly metal complexing and photodegradable EDTA. Tests were carried out at 26–27 °C and under continuous white fluorescent light of a 10 to 12 klux intensity, and a 48 h test duration. Concentrations resulting in 50% reduced growth rate (EC50) were for *C. calcitrans*, *Chlorella sp.* and *D. tertiolecta,* respectively: Cd in artificial seawater; 3.28, 0.74, and 25.6 mg L^{-1} , and in natural seawater; 3.02, 0.32, and 34.6 mg L^{-1}. EC50 values for Pb in artificial seawater were 1.4, 0.12, and 5.25 mg L^{-1} d and in natural seawater 0.18, 0.4, and 6.77 mg L^{-1}. Pb was consistently more toxic to the algae than Cd, and *Chlorella sp* was generally most sensitive followed by *C. calcitrans* while *D. tertiolecta* was the least sensitive. Toxicity levels in the natural and synthetic seawater media were similar except for Pb toxicity with *C. calcitrans,* which was more sensitive in natural seawater than in the synthetic medium. The test medium contained a minimum amount of iron and chelator and it appears to have worked although the medium may not be stable in the long-term, which could have been achieved with a large chelator surplus.

INTRODUCTION

Industrial and municipal waste discharges will frequently contain heavy metals, which may lead to temporary as well as long-term contamination of aquatic environments. This can generate problems although under some circumstances metals become immobilized or transformed into nonbioavailable forms limiting their toxicity. Toxicity and environmental behaviour of heavy metals are highly dependent on their physico-chemical speciation, which is metal and environment specific. Toxicity bioassays can be a valuable for determining bioavailability and can also be cost effective for screening and monitoring metal containing waste waters.

Algal toxicity tests can also be useful both as regulatory tools and for the purpose of providing information not only on toxic effects on phytoplankton communities, but also in the establishment of safe environmental concentrations that ensure protection of the ecosystem. Basic scientific information is needed on the toxicity levels of metals towards marine phytoplankton. In addition, cost-effective marine algal test protocols for metals are needed for tracing and monitoring sources of metal pollution and metal contamination of the environment.

For marine environments few algal toxicity studies with heavy metals have been conducted using methods of sufficient sensitivity to reflect environmental conditions and at the same time using internationally harmonized protocols. Standard marine algal test protocols (ASTM, 1990; ISO, 1995) were developed in a rush as there was a need for use with notification of chemicals and the use of large EDTA chelator concentrations have been prescribed. While this may be acceptable for the testing of organic chemicals, it rules out any environmentally meaningful testing of metals.

There is very little known on the toxicity of metals to tropical phytoplankton. In this study the following three species were chosen as test organisms, *Chaetoceros calcitrans*, *Chlorella* sp. and *Dunaliella tertiolecta*. The aim was to establish toxicity test methods with tropical marine algae suitable for metals and tentatively to investigate Cd and Pb toxicity for tropical marine phytoplankton populations.

The focus on heavy metals as test materials was decided also with a view to anticipated pollution problems in Thailand due to past large scale tin mining activities, and in consideration also of the fact that Thailand is undergoing a rapid industrial development, which poses a risk of both diffuse and point source pollution of the environment with heavy metals and synthetic chemicals. For these reasons it was considered an appropriate goal for a Thai-Danish cooperative research project to develop a marine algal test for tropical conditions that would be suitable for test materials containing heavy metals. Such a test could be a useful instrument in future pollution control in Thailand and would also be the basis for an initial assessment of which metal levels would be toxic to marine phytoplankton (and hence marine life) in Thai waters.

Lead and cadmium were selected as model or reference toxicants because these elements show very different physical chemical behaviour and at the same time they are common or typical constituents of heavy metal pollution. Lead is readily complexed and binds strongly to dissolved and particulate organic matter and only a minor portion of lead in seawater is therefore present as freely dissolved ions. Cadmium to the contrary tends to be freely dissolved as cadmium ion and chloride complexes. Cadmium therefore serves as an easily tested reference compound in bioassay development while lead is much more difficult but as such suitable for assessing the limits of various test parameters and for checking out procedures. At the same time, data for the toxicity of the two metals towards marine phytoplankton species generated by a "new generation" method has immediate application in pollution risk assessment.

MATERIALS AND METHODS

The toxicity test procedure was a modification of the International Standard: ISO 10253 "Water quality-Marine algae growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*". In order to make the test suitable for metals, the concentrations of iron, chelator and micro elements in the medium were reduced to limit the quantity of added components that can interact with the tested metals (Jensen *et al.*, 1997). Iron was added to a low but sufficient concentration to maintain exponential growth in controls for at least 24 h.

Chelator was added in stoichiometric proportion to iron plus "heavy trace elements". The objective was to have a relatively small chelator concentration to avoid masking toxicity of the tested metal, but at the same time to have enough chelator to prevent precipitation of iron. The simple stoichiometric balancing of the two major components iron and chelator proved to work in practise although the medium may not be stable in the long-term as may have been the case with a larger chelator surplus. While the chelator of the ISO medium is EDTA, citrate was used in this study to avoid problems with photodegradation of Fe(III) EDTA (Nutsch personal communication, Jensen *et al.* unpublished). Citrate was also considered more environmentally relevant than EDTA for metal toxicity testing, because EDTA has a high affinity for metals other than iron and also bonds to the macro ions Mg^{++} and Ca^{++}, which are present at high concentrations in seawater. Being a generally weaker chelator than EDTA, citrate is believed to better simulate natural chelating compounds than EDTA does.

Stock cultures of marine phytoplankton were grown in 250 ml Erlenmeyer flasks in natural seawater using the same medium as in the test (Table 1). Fe-citrate, however, was added at a four times higher concentration in the stock cultures compared with the test. A preculture was incubated for 3 days under test conditions prior to using the algae for the actual test. This procedure eliminates any lag phase and normally also safe-guards against potential culture synchronization. Stock solutions of Cd and Pb were prepared from

Table 1 Nutrient stock solutions

Nutrient	Concentration in stock solution	Added volume to test medium	Concentration in finished medium
Stock solution 1a: Fe-Citrate			
$FeCl_3.6H_2O$	0.0149 g/100 ml	1 ml /l medium	0.149 mg/l
Citric acid mono hydrate	0.0576 g/100 ml		0.576 mg/l
Stock solution 1b: Micro nutrients (trace metals)			
H_3BO_3	185 mg/l		185 µg/l
$MnCl_24H_2O$	415 mg/l		415 µg/l
$ZnCl_2$	3.0 mg/l	1 ml /l medium	3.0 µg/l
$CoCl_22H_2O$	1mg/l		1.5 µg/l
$CuCl_22H_2O$	0.01mg/l		0.01µg/l
$Na_2MoO_42H_2O$	7.0 mg/l		7.0 µg/l
Stock solution 2: Vitamins			
Thiamin hydrochloride	250 mg/l		25 µg/l
Biotin	0.05 mg/l*	0.1 ml /l medium	0.005 µg/l
Vitamin B_{12}(Cyanocobalamin)	0.50 mg/l*		0.05 µg/l
Stock solution 3: Macro nutrients			
KH_2PO_4	1.92 g/l		1.92 mg/l
KNO_3	59.5 g/l	1 ml /l medium	59.5 mg/l
Na_2SiO_3	8.57 g/l		8.57 mg/l

** Can also be made with 0.001 g biotin + 0.01 g B_{12} to 100 ml, and take 5 ml of this solution to 1 l of the Thiamin hydrochloride solution.*

All the stock solutions are made in acid washed glass ware, and Ultra High Quality(UHQ) water must be used for dilution.

Table 2 Artificial sea water [ISO 10253]

Salt	Concentration of salt in artificial sea water [g/l]
NaCl	22.0
$MgCl_2.6H_2O$	9.7
Na_2SO_4(anhydrous)	3.7
$CaCl_2$ (anhydrous)	1.0
KCl	0.65
$NaHCO_3$	0.20
Salts of H_3BO_3	0.023

Make up the artificial sea water to 1000 ml with UHQ water

$CdCl_2.2H_2O$ and $Pb(NO_3)_2$, respectively, in deionized water.

The test was conducted in both artificial seawater (Table 2) and natural seawater (salinity = 30 ppt), which was filter-sterilized through 0.2 µm pore size cellulose membrane filters. The media were prepared on the day of the test, and aerated for at least 1 h to obtain equilibrium between air and the carbonate buffer system. The test was carried out using a mini scale version of the ISO standard (ISO, 1989) as described by Arensberg *et al.* (1995) using acid washed 20 ml glass scintillation vials as test containers and 10 ml liquid volume.

Dilution series of the test material were made to establish concentration-response relationships. For preliminary tests the following logarithmic dilution series; 0.1, 0.32, 1.0, 3.2 and 10.0 mgl^{-1} were used for both Cd and Pb. Six control replicates (containing only algal medium) and three replicates per concentration were used. Definitive concentration ranges were determined on the basis of results obtained from the preliminary test. Definitive tests were then carried out twice. The

pH of the test media was regulated to about 8.3 by addition of HCl or NaOH when necessary. When the tests were finished, pH was measured again. The variation of pH was limited to a range of ± 0.3 pH unit. The concentration of Cd and Pb in the test solution were determined with Electrothermal Atomic Absorption Spectrometer (ET-AAS) after an extraction by APDC/MIBK technique (APHA, 1992).

The test volume was 10 mL. Each phytoplankton species was inoculated to the same biomass (about 0.26 mg/L), referenced as ash free dry weight. Because of different cell sizes the inoculated cell numbers differed as follows: *Chaetoceros calcitrans*: 10^4 cell/ml, *Chlorella* sp.: 510^4 cell/ml and *Dunaliella tertiolecta*: 310^3 cell/ml. The vials were incubated for 48 h on a microplate shaker at 200 rpm under continuous white fluorescent light of intensity approx. 10 to 12 k lux and temperature between 26 ± 2°C. Vials were illuminated from below eliminating light scatter from the lids.

Biomass measurements were performed at 0, 24 and 48 hours as measurements of fluorescence on extracted pigments (described below; modified from Mayer *et al.*, 1997), and average growth rates were calculated by linear regression of log biomass versus time. At time zero the vials were first exposed to the test condition for about 30 min before samples were extracted allowing the fluorescence to reach its maximum level.

For extraction the algal test suspension (one mL) was transferred directly from the vials to a screw cap test tube (size, 16*100 mm) followed by the addition of 4 ml of acetone and a drop of saturated $MgCO_3$ solution. The tube was then closed, immediately shaken and then left in a dark at 4°C until fluorescence measurements could be carried out (24 h for *C. calcitrans* and *Chlorella* sp. and 48 h for *D. tertiolecta* which was more difficult to extract). Before measurement the tubes were centrifuged at 3000 rpm for 10 min.

The fluorescense data were used directly for establishing a concentration-response curve. The Weibull equation (Christensen and Nyholm, 1984) was fitted to the data by weighted non-linear regression using a computer program developed by the Technical University of Denmark (Andersen, 1994). The program uses the "Downhill Simplex method" for a weighted least squares fit to replicate means. Weighting factors are inversely proportional to the empirical variance as estimated from replicates. Confidence intervals around EC-values were calculated by inverse estimation after a Taylor expansion.

RESULTS

The estimated EC values for 10, 20 and 50% reductions of the average specific growth rate by Cd and Pb are shown for *C. calcitrans* in Table 3, for *Chlorella* sp. in Table 4, and *D. tertiolecta* in Table 5. These tables also include measured pH variations and control growth rates.

The rapid growth of *C. calcitrans* (μ = 2.0–2.7 d^{-1}, Table 3) resulted in a high CO_2 consumption generating a pH drift of 0.4–0.6 pH units. To avoid this problem in the future it is suggested to shorten the incubation period to 24 h or reduce the size of the inoculum. Drift of pH was not a problem for the slower growing *Chlorella* and *Dunaliella*, which only fluctuated 0.1–0.2 pH units (Table 4 and 5).

Table 3 Cadmium and lead toxicity bioassays using *C. calcitrans* in artificial and natural sea water. pH levels at the end of the experiments, average control growth rate and average EC10–EC50 values are shown.

	Cadmium		Lead	
	Artificial Sea Water	Natural Sea Water	Artificial Sea Water	Natural Sea Water
pH	8.1–8.7	8.2–8.8	8.4–8.9	8.4–8.8
μ [d^{-1}]	2.04–2.05	2.07–2.35	2.52	2.69
EC_{10} [mg/l]	1.31	0.98	0.29	
EC_{20} [mg/l]	1.86	1.51	0.52	
EC_{50} [mg/l]	3.28	3.02	1.40	1.76

Table 4 Cadmium and lead toxicity bioassays using *Chlorella* sp. in artificial and natural sea water. pH levels at the end of the experiments, average control growth rate, and and average EC10–EC50 values are shown.

	Cadmium		Lead	
	Artificial Sea Water	Natural Sea Water	Artificial Sea Water	Natural Sea Water
pH	8.2–8.3	8.2	8.2–8.3	8.3–8.4
μ [d^{-1}]	1.20	1.22	0.84	0.77
EC_{10} [mg/l]	0.19	0.13	0.01	0.06
EC_{20} [mg/l]	0.31	0.18	0.02	0.12
EC_{50} [mg/l]	0.74	0.32	0.11	0.40

Table 5 Cadmium and lead toxicity bioassays using *D. tertiolecta* in artificial and natural sea water. pH levels at the end of the experiments, average control growth rate, and average EC10–EC50 values are shown

	Cadmium		Lead	
	Artificial Sea Water	Natural Sea Water	Artificial Sea Water	Natural Sea Water
pH	8.2–8.3	8.1–8.4	8.3–8.4	8.3
μ [d^{-1}]	1.13	0.81	0.59	0.81
EC_{10} [mg/l]	5.44	19.4	3.24	0.012
EC_{20} [mg/l]	8.96	24.1	3.85	0.060
EC_{50} [mg/l]	25.6	34.6	5.25	6.77

Table 6 Summary of cadmium and lead toxicity bioassays with *Chaetoceros calcitrans*, *Chlorella* sp. and *Dunaliella tertiolecta* in artificial and natural seawater. Average EC-50 values with 95% confidence intervals (mg Cd or Pb/l).

	Cadmium		Lead	
Plankton Species	Artificial Seawater	Natural Seawater	Artificial Seawater	Natural Seawater
C. calcitrans	3.28 ± 0.16	3.02 ± 0.29	1.40 ± 0.51	0.18 ± 0.01
Chlorella sp.	0.74 ± 0.40	0.32 ± 0.04	0.12 ± 0.10	0.40 ± 0.40
D. tertiolecta	25.6 ± 7.2	34.6 ± 4.0	5.25 ± 13.0	6.77 ± 2.75

A summary of the cadmium and lead EC-50 values and the confidence intervals for the studied phytoplankton species are shown in Table 6. For cadmium there was no or only minor difference between the artificial and natural seawater. There were considerable interspecies differences in sensitivity to Cd with *Chlorella* being the most sensitive (EC-50 = 0.3 and 0.7 mg Cd/l) followed by *Chaetoceros* (3.0 and 3.3 mg Cd/l) and *Dunaliella* (26 and 35 mg Cd/l).

For lead a statistically significant difference between artificial and natural seawater was observed for *Chaetoceros*, which was more sensitive in natural seawater than in artificial seawater, but otherwise the results did not differ between the two metals. Also for Pb there was considerable inter-species differences in sensitivity and the order of sensitivity was the same. *Chlorella* was again the most sensitive species (EC-50 = 0.12 and approximately 0.4 mg Pb/l), followed by *Chaetoceros* (0.2 and 1.4 mg Pb/l) and *Dunaliella* (5.3 and 6.8 mg/l) for artificial and natural seawater respectively.

DISCUSSION

The toxicity of heavy metals to marine phytoplankton has rarely been determined and data are scarce especially for tropical species. A further issue is that most older phytoplankton bioassay have been characterized by lack of comparability of test methods and principles. The need for standardization and control of physical, chemical, and biological variables during algal bioassays were outlined for metals by Peterson and Nyholm (1993) and general toxicity testing was discussed by Nyholm and Kallqvist (1989) and Nyholm and Peterson (1997).

There are several factors in making algal bioassays suitable for metal testing, which can be summarized as, any factor that will affect the speciation of the metal will also affect toxicity. It is therefore essential to control such factors as much as possible. This includes controlling pH fluctuations and the release of dissolved organic material during the test, which can be achieved by the use of dilute algal cultures with adequate mixing. In addition it is essential to limit excess organic chelators in the medium, which at the same time keeps iron available. The concentration of iron in the medium needs to be complimented with an equimolar amount of chelator, such as EDTA or citric acid; if the chelator is present in large excess then test metals will be complexed and rendered less toxic.

A standardized protocol (ISO 1997) was modified to allow for measurements of metal toxicity. The ISO standard (ISO 1997) allows a maximum pH drift of as much as 1.5 unit, which is clearly unacceptable for toxicity assessments of test material containing heavy metals. In this study the pH variations in tests with *Chlorella* sp. and *D. tertiolecta* were indeed very narrow while a pH drift of 0.5–0.6 unit occurred in tests with *C. calcitrans* due to the rapid growth of this species (average μ = 2.0–2.7 d^{-1}) which results in a high CO_2 demand towards the end of the test. For comparison the growth rate of *Chlorella* sp. was: μ = 0.77–1.22 d^{-1} and for the large *D. tertiolecta* the growth rate was only 0.59–1.13 d^{-1}. The tests used here were sufficiently robust for algae with a growth rate up to 1.2 d^{-1} while for growth rates above 2.0 the pH drift was significant. For such high growth rates lowering the initial cell density or decreasing the incubation time are two options that would decrease the pH drift to acceptable levels and at the same time extend the exponential growth at maximum rate throughout the entire test period.

Iron also plays a crucial role in this because it is difficult to keep iron in solution in seawater (synthetic seawater in particular). Iron is an essential element for algal growth, but interacts with the expressed toxicity of heavy metals through competition for chelators. Media are not stable in the long run, unless iron is maintained dissolved by adding a large excess of chelator as prescribed in standard test protocols. The reason for this is competition for iron with other complexers, hydroxyl ions in paticular, as well as with various precipitation reactions. The added chelator may become tied up by binding to other metals including Mg^{++} and Ca^{++}, which are major constituents of seawater. Nevertheless, it was found that simply by adding iron and chelator in equimolar amounts to the level used in standard protocols for tests with freshwater algae was an option that worked in practise by supporting sufficient algal growth while limiting the amount of metal detoxification.

With respect to growth support, there was no clear difference between media based on artificial seawater and filtered natural seawater other than for *Dunaliella,* which grew fastest in natural seawater. The toxicity of Cd was similar in both artificial and natural seawater for all the phytoplankton species. For lead there was also little difference between natural and artificial seawater for *Chlorella* sp. and *D. tertiolecta.* However, *C. calcitrans* showed an order of magnitude higher sensitivity in the filtered natural seawater compared with artificial seawater.

These results can be explained in part by simple physical chemistry (Stumm and Morgan, 1981). The major species of Cd in seawater (pH around 8.2) are chloride complexes, $CdCl_2$ and CdCl , and Cd is only complexed to a small extent by organic matter. Pb, on the other hand binds strongly to organic material, and the major species of Pb in seawater are $PbCO_3$ and PbOH. It is therefore natural to expect Cd behaving similarly

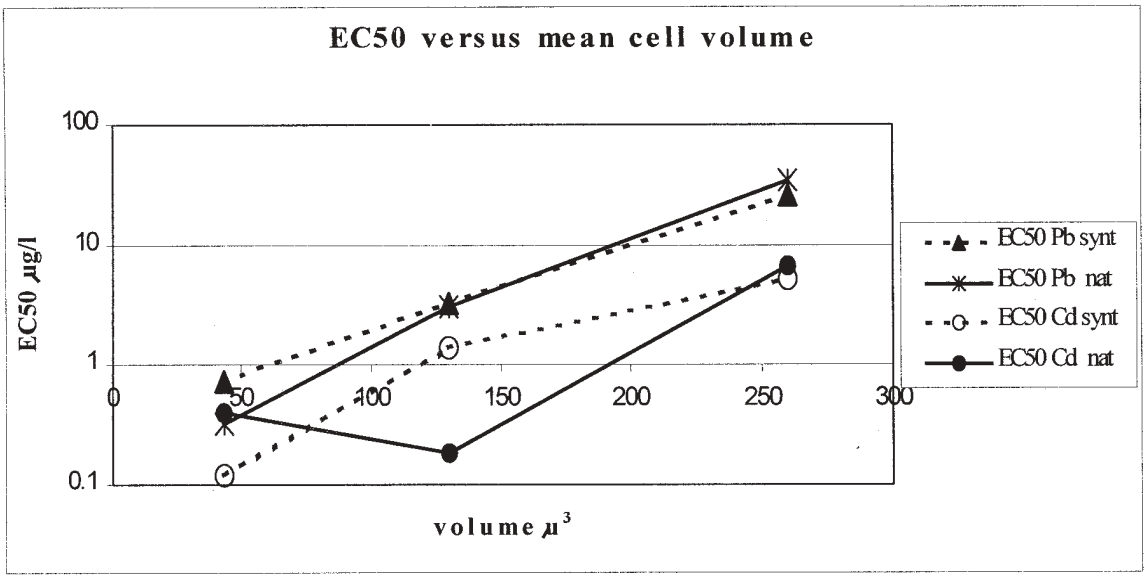

Figure 1 Relationship between EC50 levels (µg/l) for growth inhibition of lead and cadmium and mean cell volume for three tropical phytoplankton. Data for artificial and natural seawater is shown.

and exerting similar toxicity in either medium. Lead toxicity can be expected to vary considerably being influenced by binding to organic matter, by inorganic complexing and by pH sensitive complex heterogenous co-precipitation/flocculation reactions which may not be in equilibrium.

It appears that Pb was more toxic than Cd for all the tested phytoplankton strains. This is in accordance with general observations that toxicity of heavy metals tends to increase with electropositivity. With regard to marine phytoplankton Phangs *et al.* (1997) found the following toxicity sequence $Cu^{2+} > Cd^{2+} > Mn^{2+} > As^{5+}$.

Among the selected three test species *Chlorella* sp. was the most sensitive, followed by *C. calcitrans* and finally *D. tertiolecta.* This sensitivity pattern suggests that sensitivity towards metals decreases with increasing cell size (decreasing surface to volume ratios) although this limited study does obviously not allow such a general conclusion. Similar size dependency has been shown for strains of *Skeletonema costatum* with different cell chain lengths for toxicity of potassium dichromate and 3,5-dichlorophenol (Hanstveit and Oldersma, 1993). For illustration of the data only, a plot of EC50 versus the mean cell volume of each species is presented in Figure 1. It is stressed that the relationship may be coincidental.

There is no information in the scientific literature describing standardized, toxicity testing with tropical marine phytoplankton species. A main objective of this work was therefore to develop a test method making routine toxicity testing possible. The developed method can be used in future marine pollution control work in tropical countries.

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