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Toxicity of Sediment from a Mining Spill to *Cylindrotheca closterium* (Ehremberg) Lewin and Reimann (Bacillariophyceae)

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On April 25th 1998 a holding pool containing acid sludge from the Aznalcóllar mine (owned by the Canadian-Swedish Company Boliden Ltd.) accidentally poured on the Guadiamar River (a tributary of the Guadalquivir River). Around five million cubic meters of sludge polluted with heavy metals, mainly Zn, As, Pb, Cu and Cd (Blasco et al., 1999) were spilled to Guadiamar River within hours. Implications of this spill to environment and human health have been studied by chemical analysis and bioassays (Suñer et al., 1999). Toxicity tests involving sediments and benthic microalgae are absent from current guidelines. Recently, bioassays using benthic microalgae (*Cylindrotheca closterium*, formerly *Nitzschia closterium*) have been designed (Moreno-Garrido 2003a, b). Microphytobenthos has a relevant role in the coastal and mudflats shelf systems (Light and Beardall 2001). Primary productivity and O_2 production at the upper level of intertidal sediment are in great part due to microphytobenthos. In some of these biocenosis, biomass of microalgae matches or even exceeds bacterial biomass (La Rosa et al., 2001). Moreover, microphytobenthos (constituted mainly by diatoms and cyanophytes) (Delgado, 1989) is the major food supply for numerous intertidal species. Thus, it is obvious that microphytobenthos might play an important role in bioaccumulation and possibly biomagnification of contaminants through the coastal food chains (Absil et al., 1996): many studies revealed the high accumulation capacity of microalgae for heavy metals (Moreno-Garrido, 1997; Fernandez-Leborans and Novillo, 1996).*C. closterium* is a widespread benthic species of temperate marine coastal and brackish waters that grow fast in low enriched media. It has been used for evaluating EC50 of experimental (laboratory polluted) and natural marine or estuarine sediments (Moreno-Garrido et al. 2003a, b). The aim of this research was to expose a population of *Cylindrotheca closterium* to a toxic mud collected from an impacted area of the Rio Guadiamar after the Aznalcollar mining spill. It will allow us to evaluate the EC50 of this toxic mud for microalgae. This method is a suitable tool for evaluating and comparing the quality of sediment samples.

MATERIALS AND METHODS

A strain of *Cylindrotheca closterium* (Ehremberg) Lewin and Reimann (formely *Nitzschia closterium* (Ehremberg) Wm. Smith) was isolated in May 2000 from a

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coastal pond near the Institute of Marine Sciences of Andalusia (ICMAN) in Puerto Real, Cádiz (SW of Spain). Since isolation, the strain was included in the Culture Collection of Marine Microalgae of the ICMAN (CCMM-ICMAN, BIOCISE). The strain was cultured in artificial substitute ocean water (ASTM, 1975) medium during at least six months before its employment in the experiments in order to avoid possible adaptation problems that would increase the lag phase in experimental cultures. Organisms used for toxicity test were always in exponential growth phase (3-day-old-cultures).

Artificial substitute ocean water (ASTM, 1975) was used in all the experiments. For routine cultures, Guillard's f/2 medium (Guillard and Ryther, 1962) enriched with 500 μgL^{-1} SiO_2 was used. For toxicity experiments, only SiO_2 ($50\mu gL^{-1}$), NO_3^- ($6\mu gL^{-1}$) added as Na NO_3, and PO_4^{3-} ($6\mu gL^{-1}$) added as $NaH_2PO_4 \cdot 2H_2O$, were used as nutrients. This procedure was developed in order to avoid the effect of the chelator EDTA, included in the Guillard's f/2 formulation, on the toxic effect of heavy metals. The nutrient concentration in this "light" medium represents the average natural concentration of the same substances in actual locations in the Bay of Cadiz (Establier et al., 1990). Satisfactory growth of control cultures of *C. closterium* during 72h in this simplified medium was checked before experiments.

Natural clean marine sediment obtained from a non-polluted area at the Bay of Cadiz as well as toxic mud from the impacted area of the Rio Guadiamar (Riba et al. 2002) were sampled by the use of a 0.025 m2 Van Veen grab, dried (60ºC) and stored. In the clean sediment, percentage of silt-sized fraction (under 63µm) was not significant. Part of this sediment was ground in a ball mill (Fritsch, model Pulverisette 6). After grinding, the silt-sized fraction was extracted by sieving. For the natural polluted mud, silt fraction was also extracted by sieving. The different dried parts of sediments (clean sediment: silt-sized and sand-sized; toxic mud: silt-sized) were stored in plastic bag until used. Particle-size distribution in each experimental flask was kept because it influences growth of *C. closterium* cells in the experiments (Moreno-Garrido et al., 2003a, b). In order to measure sediment grain size an aliquot of wet toxic sediment was analysed using a laser particle size Fritsch (model Analysette 22), as reported in DelValls et al., 1998.

For trace metal analysis, known weights of sediments were digested following Loring & Rantala (1992). Concentration of heavy metals Cu and Zn were determined in a flame AAS (Perkin Elmer 2100); Cd and Pb were determined by graphyte furnace AAS (Perkin Elmer 4100 ZL); and As was determined by a Perkin Elmer MHS-FIAS coupled to the 4100 ZL spectrophotometer. Analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) allowing an agreement with certified values higher than 90%. PAHs and PCBs analysis were performed according to USEPA SW-846 Method 827C78082 and measured as described in Riba et al. (2002). NRC-CNRC HS-6 sediment was used as quality control for PAHs, while NRC-CNRC HS-1 was used as quality control for PCBs, expressed as AROCLOR 1242 and 1260. For both organic

chemicals (AROCLOR and PAHs), analytical procedures allowed an agreement with certified values higher than 90%.

In order to test the toxicity of the assayed mud, different percentages of dry polluted and clean silt-sized sediment were mixed with clean sand to final dry weight of 5 g, which is the minimal amount of sediment able to cover the bottom of 125 mL capacity borosilicate Erlenmeyer flasks used. For this species, maximum growth of cells is observed when sand-sized particles (> 63 μm) percentage was between 100 and 90%, in the experimental conditions. When silt-sized particles (< 63 μm) percentage was higher than 10%, a decrease of growth rate occurs (Moreno-Garrido et al., 2003 a, b). Thus, 4.5 g of clean dry sand was mixed with 0.5 g of dry silt-sized sediment. This latter was composed by clean silt-sized sediment (obtained my moulting ground in a mill) (Fritsch, model Pulverisette 6) and polluted mud, reaching different percentages respect the total weight of the experimental sediment (sand + clean silt + mud): 0; 0.05; 0.5; 5 and 10% of polluted mud. Experiments were carried out by triplicate. Afterward 50 ml of *C. closterium* culture, enriched as described above, was added to each sterile, Perlon-topped flask. Initial cellular density was 10^4 cells mL^{-1} and cellular density was measured every 24 h, for 72 h, by counting in Neubaeur chamber. This initial cellular concentration will provide a surface cellular density (when all cells settle to the sediment) of 1.8 10^3 cells cm^{-2}. This surface cellular concentration is in the range of those found in natural environments (Delgado, 1989). Cellular population growth curves were plotted, and the area under that curves and percentage of inhibition for each toxic mud concentration calculated as described in OECD (1998) protocols. Then, EC_{50}%72h was calculated following Hampel et al. (2001). Flasks were shaken only before counting (one time each day). Cells were observed under light microscopy (Olimpus, BH-2). The use of a barrier filter of 530 nm, combined with fluorescence emission in the microscopy resulted in a clearly observable bright-red fluorescence of the chlorophyll contained in chloroplasts of the diatoms, thus being easy to discriminate cells from sediment particles and to count them.

RESULTS AND DISCUSSION

Results of the analysis for the sediments (clean and contaminated) are shown in Table 1. The Aznalcóllar mining spill was characterized for a high concentration of As and Zn, although other heavy metals were also present in the holding pool and the subsequent spill. Following OECD procedures (1998) (Comparing areas under growth curves) a concentration versus inhibition graphic is plotted (Figure 1). Near 85% inhibition was obtained for the highest proportion of toxic mud (10% of total), which made possible the calculation of EC_{50}%72h value, which is, the concentration of polluted sediment that inhibits growth of the microalgal population 50% with respect to the controls after 72 h exposure.

After applying equation described in Hampel et al. (2001) in order to calculate EC50, this value was 4.3 ± 0.7 (standard error) % of polluted silt in experimental sediment. This means that a mixture of 4% of the polluted silt with clean sediment

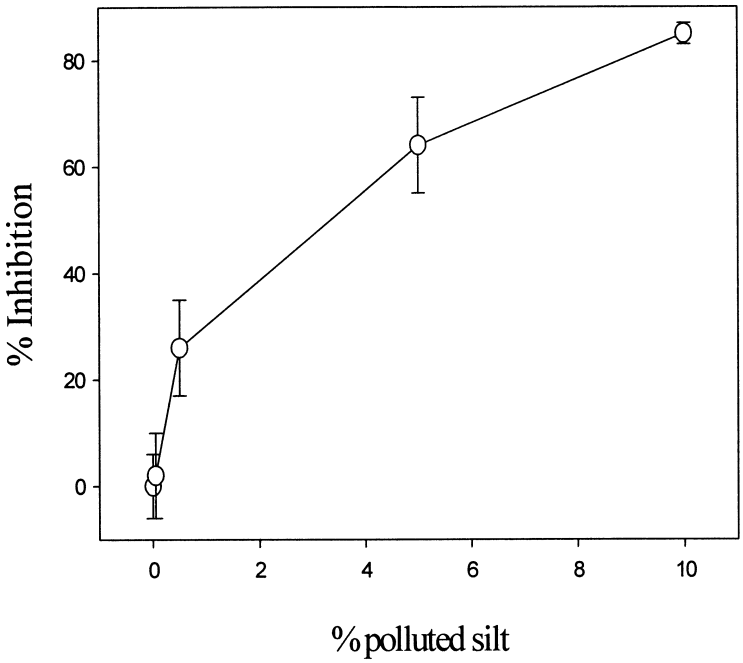

Figure 1. Percentages of polluted silt in the experimental sediment versus percentage of inhibition for microalgal populations. Bars mean standard deviations (n=3).

Table 1. Organic Carbon (O.C.) %, total PAHs, PCBs (A 1242 and A 1260) and metal concentration for As, Cd, Cu, Pb, and Zn

	Clean sediment	Polluted sediment
O.C.*	1.4	0.4
PAHs	0.30	n.d.
A 1242	0.45	n.d.
A 1260	0.09	n.d.
As	11.20	4088
Cd	0.1	45.66
Cu	9.5	2033
Pb	71.90	7873
Zn	41.6	21618

*O.C. expressed as percentage of dry weight; rest of values expressed as $mg \cdot kg^{-1}$ dry sediment; n.d. means non detectable concentrations.

inhibits of the exposed population of *C. closterium* 50% with respect to the controls. In terms of metal concentration for the studied metals, this means 176 mg kg^{-1} for As, 2 mg kg^{-1} for Cd, 87 mg kg^{-1} for Cu, 338 mg kg^{-1} for Pb and 930 mg kg^{-1} for Zn.

Bioassays performed on *C. closterium* are not more sensitive than those designed for amphipods (*Corophium volutator* and *Ampelisca brevicornis*) if mortality of the animals (LD50% for ten days) and growth inhibition of the microalgae (EC50% 72 hours) are compared. Both amphipods showed mortalities of near 90% when exposed for ten days to a mix of 1.8% polluted sediment with clean sediment in the experimental conditions (Riba et al., 2003).

Polluted silt percentages of 0.3% produced in the assayed animals mortalities of 20-40% for *C. volutator* and 5-25% for *A. brevicornis*. Those values are quite similar to the 17-35% of growth inhibition reached for a dilution of 0.5% for polluted sediment in the experiment described in this paper.

In tests performed on flat fishes (*Solea senegalensis*) and clams (*Scrobicularia plana*) for the same sediment, assays for 15-30 days and 14 days (respectively) resulted in semi-quantitatively detectable lesions in different tissues and organs such as liver, gills, gut or kidney for *S. senegalensis*, gill and gut for *S. plana*. Most of the lesions began to be detectable in both organisms at a dilution of 0.3% of polluted sediment (Riba et al., 2004).

The use of benthic microalgae (microphytobenthos) in sediment toxicity tests is a recent topic in ecotoxicology in spite of the importance of this group in coastal environments. Knowledge about sensitivity of the organisms at the base of the trophic chain to pollutants adsorbed to sediments should be taken into account if environmental quality assessments are done.

The test described for *C. closterium* is quick and cost-effective. Availability of tests organisms in microalgal bioassays does not depend on seasonality, and are highly repeatable. Additionally, special equipment is not necessary in the lab to perform the described bioassays. In the case of the studied sediments, sensitivity is comparable to the sensitivity reached in bioassays involving other organisms (amphipods, molluscs or fishes), but results were obtained after just 72 h.

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