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Marine Benthic Microalgae *Cylindrotheca closterium* (Ehremberg) Lewin and Reimann (Bacillariophyceae) as a Tool for Measuring Toxicity of Linear Alkylbenzene Sulfonate in Sediments

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Organisms belonging to several taxonomic groups have been used in order to detect and measure toxicity of different substances in benthos (SETAC 1993). Recently, efforts have been made in standardization of suitable organisms able to be used in sediment toxicity tests. Related to this subject, microalgae have not received much attention, even taking into account the relevant role of this group in benthic environments. This is especially noticeable in mudflats and coastal shelf systems (Light and Beardall 2001; Blanchard et al. 2000), where biomass of microalgae can match or even exceed bacterial biomass in intertidal sediments (La Rosa et al. 2001). References of sediment toxicity test involving benthic microalgae have been found in the literature (Moreno-Garrido et al. 2001).

The diatom *Cylindrotheca closterium* is a very widespread benthic species, that demonstrated fast growth in laboratory cultures, even in poorly enriched media. Taking growth inhibition as the endpoint, a novel approach to sediment toxicity testing has been developed, by exposing benthic microalgal populations to laboratory sediment spiked with linear alkylbenzene sulphonate (LAS), a synthetic organic surfactant produced in large quantities (Tolls et al. 1997). Surfactants can alter membrane permeability, enzyme and lysosomal activity and tissue structure in organisms, inducing alterations in electron-transfer chains (Argese et al. 1994; Blasco et al. 1999; Bragadin et al. 1996; Lewis 1992). Surfactants tend to accumulate in high quantities in urban-polluted coastal sediments (González-Mazo et al. 1997).

MATERIALS AND METHODS

A strain of *Cylindrotheca closterium* (Ehremberg) Lewin & Reimann (Bacillariophyceae), formerly *Nitzschia closterium* (Ehremberg) W. Smith, was isolated in May 2000 from a salt pond in Puerto Real (Cádiz, SW Spain). A SEM micrograph of the microalgal cells can be seen in Figure 1. Since isolation, the strain was included in the Culture Collection of Marine Microalgae of the Marine Science Institute of Andalucía (CCMM-ICMAN). The strain was cultured in artificial substitute ocean water (ASTM 1975) medium during six months before its employment in the experiments in order to avoid possible adaptation problems that would increase the lag phase in experimental cultures. Microalgal cells used

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for toxicity tests were always in exponential growth phase (three-day-old cultures). For routine cultures, Guillard's f/2 medium was used (Guillard and Ryther 1962). For toxicity experiments, a simplified nutrient medium with the following composition was used: SiO_2 (50 μgL^{-1}), NO_3^- (6 $\mu g\ L^{-1}$) and PO_4^{3-} (6 $\mu g\ L^{-1}$). These major nutrient concentrations were selected because they are similar to the average natural concentrations measured in actual locations in the Cádiz Bay (Establier et al. 1990), and thus possible interferences of nutrient excess on behaviour of toxic substances are avoided while increasing the ecotoxicological relevance of the test. Satisfactory growth of control cultures of *C. closterium* during 72 hours in this medium was checked.

Natural marine sand obtained from a low-polluted area (Sancti Petri, SW of Spain) was washed in the laboratory with 0.1 N HNO_3, and rinsed with ultra-pure water (Milli Q) several times. Aliquots of this material were moulted in a mill (Fritsch, model Pulverisette 6). The silt-sized fraction (<63 μm) was not significant in the initial sediment. After grinding, more than 95% of the sediment was silt-sized.

A weight of 10 g of this silt-sized sediment was exposed over one week to 1 L substitute ocean water with a concentration of 10 mg L^{-1} of commercial LAS, at 20 °C. Commercial LAS was supplied by Petroquímica Española, S.A. (PETRESA). Molecular weight of the mixture was 342.4 u.a.m. After spiking, the sediment was separated from the supernatant by decantation and washed with 1 L of ultrapure (Milli-Q) water, in order to remove remaining salt and non-adsorbed surfactant. Resultant sediment was separated from the supernatant by decantation and dried overnight at 60 °C. Levels of LAS in dry sediment were measured by HPLC (courtesy of PETRESA).

An experiment was designed in order to determine the effect of size distribution of the sediment on microalgal growth. On a total weight of 5 g (minimum amount of sediment able to cover the bottom of the borosilicate conical flasks used in all experiments), increasing percentages of silt-sized sediment were mixed with sand-sized sediment. After this experiment, a fixed proportion of 10% silt-sized versus 90% sand-sized sediment was used, which permitted optimal growth. The 10% silt-sized sediment was, thus, composed by different proportions of clean and polluted silt-sized sediment, in order to maintain the granulometry of the different treatments. A first wide-range experiment revealed that sediment obtained by the method described above was highly toxic to *C. closterium*. Thus, a ten-fold dilution of this material was performed with clean (non-spiked) silt-sized sediment before a fine-range experiment.

Sediment toxicity tests were performed in conical 125 mL capacity sterile borosilicate conical flasks, topped with synthetic cotton (Perlon). On 5 g of sediment with increasing concentrations of LAS, 50 mL of *C. closterium* culture, enriched as described above, with initial cellular concentration of 10^4 cells mL^{-1} was added. Cultures were shaken daily and cellular density counted under light microscopy on a Neubauer counting chamber. Taking into account population

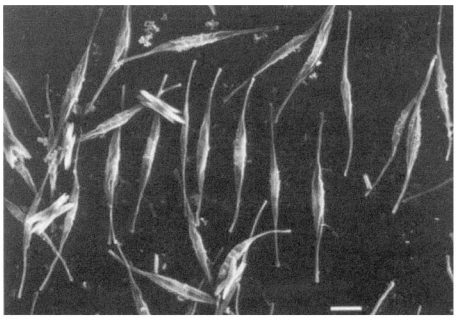

Figure 1: Scanning electron microscopy of *Cylindrotheca closterium* cells. Scale bar is 10 μm. Mean apical axis length is around 60 μm.

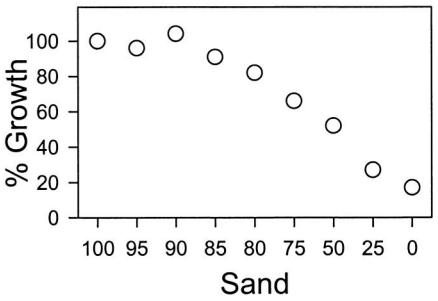

Figure 2: Percentage of growth related to 100 % sand when percentage of silt-sized sediment (100 - sand percentage) increases

growth data and following OECD procedures (OECD 1998), growth curves were plotted, area under the plotted curves calculated and growth inhibition percentages established, by comparison of the area under the growth curve of control populations. Resultant data were fitted to a sigmoid model and EC50 calculated following Hampel et al. (2001).

RESULTS AND DISCUSSION

Granulometry affects population growth of *C. closterium* under experimental conditions. Increases in silt-sized material percentages inhibit optimal growth. Optimal growth occurs when populations of microalgae used are exposed to 100% sand-sized sediment, as can be seen in Figure 2. A one-way ANOVA ($p < 0.05$, method Fisher's LSD) was performed in order to determine the maximum percentage of silt-sized sediment that permitted a cellular growth statistically not distinguishable from controls (assumed as 100% sand). This percentage was 10% silt-sized sediment vs. 90% sand-sized sediment. This size distribution was used in all further experiments with spiked sediment.

This growth behaviour can be explained by the size of the cells of *C. closterium* (around 50 μm of apical axis). Most of the particles forming silt-sized sediments are smaller than this microlagal species. Thus, a slower settling of particles occurs

Table 1: LAS homologue distribution in commercial LAS, non-spiked and spiked sediment. Source: PETRESA.

Homologue distribution (%)	C10	C11	C12	C13
Commercial LAS	10.9	35.3	30.4	21.2
Non-spiked sediment	10.7	32.7	29.1	27.5
spiked sediment	14.3	35.5	30.9	19.3

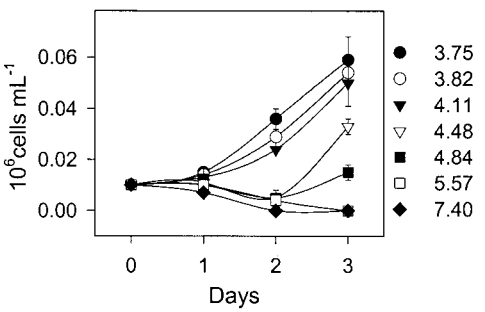

Figure 3: Growth curves of *C. closterium* cultures exposed to sediment with increasing levels of LAS expressed as mg kg $^{-1}$. Error bars mean standard deviation between triplicates.

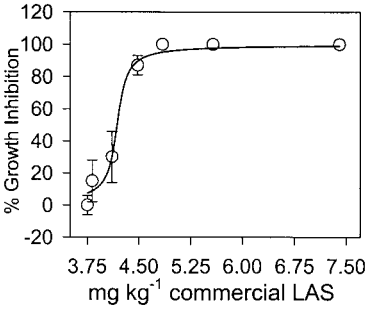

Figure 4: Growth inhibition percentages respect the control of *C. closterium* cultures versus increasing LAS concentrations in sediment and fitted to a sigmoid curve. Error bars mean standard deviation between triplicates

and a shadowing effect happens when particles set over microalgal cells, reducing photosynthesis. Possibly, the energy needed to unbury, in addition to longer times under a silt-sized sediment layer (out from the photic zone) will determine smaller growth rates. But even in the worst case (100% silt-size sediment), cells increase more than four fold in 72 hours showing active behaviour under microscopy. At 50% silt-size vs. sand-size sediment, this increase from initial cellular density is around twelve fold, being quite different from the forty five fold in 100% sand sediments (data not shown). Improvements in the design of the tests can be made by the selection of smaller microalgal benthic diatoms (i.e., *Navicula spp.*), which would settle slower than silt sized particles and thus would avoid shadowing problems of the cells by smaller sediment.

Analysis of different LAS homologues in spiked and non-spiked sediments is shown in Table 1. Original spiked sediment reached a concentration of adsorbed LAS of 733 mg LAS kg^{-1}. Levels of LAS in supernatant after 72 h were not measured. Control and six increasing concentrations of adsorbed LAS (after diluting the spiked material with clean silt-sized sediment) resulted in different growth curves (Figure 3). After calculation and comparison of the area under each growth curve with area under the control growth curves, percentages of growth inhibition were calculated. These results were fitted to a sigmoid curve and EC50 calculated following Hampel et al. (2001) (Figure 4), resulting in a value of 4.18 ± 0.03 (standard error) mg LAS kg^{-1}.

Literature about bioassays involving adsorbed LAS in sediments is very scarce, and the results obtained in the reviewed works revealed higher resistance of other organisms when compared with microalgae. Levels of 25.87 mg LAS kg-1 did not affect *Branchiura sowerbyi* (Oligochaeta, Tubifidae) (Castellato et al. 1992). Marin et al. (1994) did not detect negative effect on mussels (*Mytilus galloprovincialis*, Mollusca) exposed to 132 mg LAS kg^{-1}. Ascidians were sensitive to dissolved LAS: 0.25-0.5 mg L^{-1} affected growth (Marin et al. 1991),

but no data were found for sediments. In soils, adults of *Folsomia fimetaria* (Collembola, Isotomidae) were not affected by levels as high as 1000 mg LAS kg^{-1}, although the EC10 for growth of juvenile animals was 163 mg LAS kg^{-1} (Holmstrup and Krogh 1996).

The difference between the level of LAS measured in non-spiked sediment (3.7 mg LAS kg^{-1}) and EC50 is very small. Probably, LAS affecting microalgae would be the spiked LAS, while LAS in non-spiked sediment must be non-bioavailable for cells. Related to this, sensitivity of *C. closterium* to LAS would be even higher than the sensitivity reported in this work.

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