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Effect of sediment turbidity and color on light output measurement for Microtox® Basic Solid-Phase Test

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Abstract

In this work, sediment samples collected from several Spanish harbours were tested with two toxicity procedures, designed for solid samples: the Microtox® Basic Solid-Phase Test (BSPT) and a modified procedure of the previous test protocol (mBSPT). According to the BSPT procedure, after initial light readings, pure bacteria were exposed to sediment suspension dilutions and light production was directly measured on suspended sediments without any further manipulation. As measurements are likely to be affected by sediment turbidity and color, a variation in initial light measurement has been here suggested, in order to consider the sample effect at all time readings during the test. Firstly, when sediment suspensions at different concentrations were added to bacteria suspension, immediately the initial light output drastically decayed by more than 50% in signal difference, resulting in a false inhibition, as effect of sample turbidity/color. This effect was more evident at high EC50 values, when slightly or not toxic samples were assessed. Secondly, the comparison of the EC50 obtained with both procedures, demonstrated that the mBSPT produced higher EC50 values (less toxic) than those obtained with the standard procedure. The mBSPT procedure resulted rapid and effective and it could be applied simultaneously with BSPT, in order to better evaluate the toxicity.

Keywords: Sediments; *Vibrio fischeri*; Bioassay; Modified test procedure; Ecotoxicology

1. Introduction

The Microtox® system is a microbial bioassay based on the natural bioluminescence of the marine bacteria *Vibrio fischeri* and used to assess the acute toxicity on environmental samples. Over the last years (Cook and Wells, 1996; Johnson, 1997; Ringwood et al., 1997; Dorn and Salanitro, 2000; Harkey and Young, 2000; Stronkhorst et al., 2003), rapid screening of sediment acute toxicity has been measured with Microtox® Solid-Phase Test (SPT) (Azur Environmental, 1995), in which the bioluminescent bacteria are exposed to a sediment suspension, and the effects on light emission are

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evaluated in the liquid phase that remains after removal of the sediments by filtration. The major confounding factor in the Microtox® SPT is the sediment texture, that influences light output readings: as the filtration protocol causes a bacteria loss due to their adhesion onto the sample matrix, a lower light emission is recorded (Benton et al., 1995; Ringwood et al., 1997). Many researchers (Onorati et al., 1998; Pellegrini et al., 1999; Lahr et al., 2003; Stronkhorst et al., 2003) applied correction procedures, based on statistical methods, to correct the particle effect on toxicity measures. For accurate toxicity assessment of whole sample, direct contact between the test organism and sediment particles is fundamental, since the toxic effect depends mostly on particle-bound compounds (Harkey and Young, 2000).

The Basic Solid-Phase Test (BSPT) is a new Microtox® test procedure for solid samples (Azur Environmental, 1995) which has been recently applied in several studies (Mowart and Bundy, 2001, 2002a,b; Abbondanzi et al., 2004; Iacondini et al., 2004), as a simpler and more rapid procedure than the SPT. In fact, the BSPT procedure allows *V. fischeri* to come in direct contact with the solid sample in an aqueous suspension, without any filtration step. However, the main disadvantage of this new method is due to sample turbidity, that holds on during luminometer recordings and can mask bacteria light output, producing higher toxicity values. The problem of sample turbidity effect on *V. fischeri* light emission has been already studied with different luminometer systems (Lappalainen et al., 1999, 2001), in order to improve the test standard procedure.

In this work, a modified BSPT procedure (mBSPT) was set up after a preliminary study (Guerra et al., 2004), using sediment samples from different Spanish harbours, and the obtained results compared with the standard BSPT procedure. Thus, the aim of this work is the evaluation of the effect of sediment turbidity and color on the light emission of the test organism *V. fischeri* when used in the BSPT procedure.

2. Materials and methods

2.1. Sample collection and characterisation

Twenty-two surface sediments (0–5 cm) (with silt + clay percentage and organic matter content of 70 ± 28% and 15 ± 6%, respectively) were collected from different Spanish harbours in April 2003 (Fig. 1). The contamination was very heterogeneous, depending on harbour activities and location. Harbours of Bilbao (Bi), Pasajes (Pa), Barcelona (B) and Cadiz (Ca) are characterised by cargo and passenger traffic while Cartagena (C) and Huelva (H) are mainly affected by historic

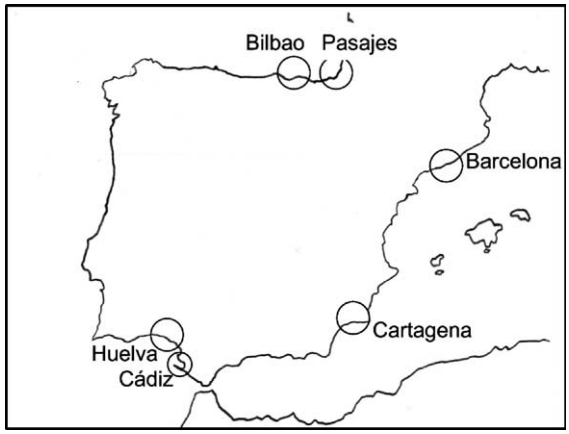

Fig. 1. Map of Spain and harbour zones.

mining activities. No contamination (chemical concentration (mg kg^{-1}): As 3.42; Cd 0.92; Cr 0.1; Cu 6.98; Hg 0.05; Ni 0.06; Pb 2.28; Zn 21.27; PCBs < 0.01; PAHs < 0.01) was detected in sample Ca1, that was used as the reference sample; whereas, different concentrations of As (3–840 mg kg^{-1}), Cr (3.5–33 mg kg^{-1}), Cu (7–1939 mg kg^{-1}), Zn (21–2458 mg kg^{-1}) and organic compounds (total PAHs up to 67 mg kg^{-1}) were detected in the other samples. Detailed protocols and results of the chemical analyses were reported by Casado-Martinez et al. (submitted for publication). Briefly, for total PAHs, EPA 8310 method by HPLC with fluorescence detection was used; for Polychlorinated Biphenyls analyses, EPA 8080 method by Gas chromatography (GC/ECD) was used, after ultrasounds extraction with cyclohexane/dichloromethane solution and clean-up. The heavy metals analyses were performed by different methods depending on the detected metal: after microwave acid digestion with HNO_3 and aqua regia 1:3 (EPA 3051A method), atomic adsorption spectrometry (for Hg and As analyses) and flame atomic adsorption spectrometry (for Cd, Pb, Cu, Zn, Ni and Cr analyses) were used.

2.2. Toxicity tests

2.2.1. Basic Solid-Phase Test (BSPT)

The Microtox® BSPT was performed according to standard operating procedure (Azur Environmental, 1995). Briefly, the end point is the inhibition of light emission by the marine bacterium *V. fischeri*. Seven grams (±0.01 g) of sediment were tested as suspensions prepared with 35 ml of diluent (35 g/l NaCl solution) and diluted to a series of nine concentrations, in the incubator wells (Microtox® model 500 Analyser). For initial light output, 510 μl of bacteria suspension was

prepared at 15 °C and, after recording I_0, the test sample (500 μl) was dispensed in the bacteria reagent. Light readings were recorded at 5, 15 and 30 min, and, in this work, only EC50 values at 30 min were reported.

Regression statistics of concentration ($\log C$) on the gamma parameter ($\log \Gamma$) were used to estimate the correlation which gives a nominal toxic effect. The software supplied by Azur Environmental calculated the EC50 value using the Γ parameter, defined as

$$\Gamma = \left[\frac{(R_t \cdot I_0)}{I_t}\right] - 1 \quad (1)$$

where I_0 = initial bacterial light emission, I_t = light emission after the introduction of the test sample at a reading time, in the bacterial suspension, and R_t = the correction factor obtained by I_t (blank)/I_0 (blank).

2.2.2. Modified Basic Solid-Phase Test (mBSPT)

When colorful or turbid samples are measured, the most important parameter to record is the signal received just after addition of the sample to the bacteria cuvette (Lappalainen et al., 2001). In this way, the effect of sample turbidity is therefore taken into account throughout the whole measurement period.

In this work, a normalization method to correct for the decreased light outputs of bacteria due to sample turbidity and color has been developed. Based on the BSPT standard procedure, the sediment sample was suspended in the diluent aqueous solution, and the *V. fischeri* light production was directly measured with the test sample at any reading time, including initial time (I_{mod}). Thus, the greatest difference between standard BSPT and modified BSPT is recorded when the sample is dispensed into the cuvette. In fact, in BSPT sample is pipetted after the initial light reading (I_0), while in just before the initial light reading (I_{mod}). Any compound present in the sample is able to produce a prompt effect on bacteria immediately after the contact with them; in fact, Lappalainen et al. (2001) reported that, immediately after the addition of bacteria into sample (0–2 s), a peak value of light production occurred, excluding any adverse effect.

After this correction, the effect concentration (EC50, mg l^{-1}) was estimated as the dry sediment concentration that causes a 50% light reduction by substituting in (1) the I_{mod} reading taken immediately after dispensing sediment dilutions into the reagent suspension for the I_0 reading taken with the bacteria only suspension. Readings at any time were taken after gently shaking the sample/bacteria suspension, in order to ensure adequate mixing during measurement according to Lappalainen et al. (2001).

BSPT and mBSPT test protocols were simultaneously applied according to following steps:

1. Measure the initial light output (I_0) of 510 μl of bacteria suspension, as outlined by BSPT.
2. Immediately, dispense 500 μl of sample suspension into bacteria cuvette and read the light output (I_{mod}), as request by mBSPT.
3. After the desired time contact to perform the acute test, gently shake the cuvette in order to re-suspend the cuvette content and read the light output (I_t).
4. Calculate the Γ parameter for BSPT and mBSPT procedure, using I_0 and I_{mod}, respectively, for initial light emission and the same I_t in formula (1).
5. When $\Gamma = 1$, the concentration of test sample applied corresponds to the EC50 value (expressed as mg dry weight l^{-1}).

3. Results and discussion

To determine if an amount of sediment toxicity observed in *V. fischeri* after exposure to sediment samples could be ascribed to sample turbidity and color, two methods of data handling were used to calculate EC50 values according to the formula reported in the Material and methods section. Sediment toxicity ranged from 100 to 20 600 mg l^{-1} (dry weight) and from 95 to 24 300 mg l^{-1} (dry weight) at 30 min exposure, when measured with BSPT and mBSPT, respectively (Fig. 2). In most cases, the calculated EC50s for the standard BSPT displayed lower values (higher toxicity) than those obtained when I_{mod} was recorded (mBSPT). In particular, out of 22 samples, 10 showed a substantial difference in EC50 values obtained with BSPT and mBSPT; in fact, the 50% effective concentrations obtained with mBSPT were found at least 15% higher than those obtained with BSPT, reaching differences of 45%, 48% and 64% for three samples (H3, H2 and B3, respectively).

The overall linear regression of BSPT versus mBSPT toxicity values (Fig. 3) resulted in a very significant correlation ($r^2 = 0.9913$ and $r^2 = 0.9707$ with and without the highest EC50 value, respectively) and confirmed the previous result. The angular coefficient in linear regression was >1, pointing out that the EC50 values obtained with mBSPT were mostly higher than those obtained with BSPT. Moreover, this regression shows that the higher the EC50 values obtained with BSPT, the higher the difference between the EC50 values.

Differences in EC50 values were produced by effect of sample concentration on initial light emission, and an example of this effect is presented in Table 1. Out of 22 sediment samples, 10 samples were chosen in role of test sample concentration. When colorless suspension was used (control, 35‰ NaCl), the initial light output (I_0) was affected by −5% due to the effect of

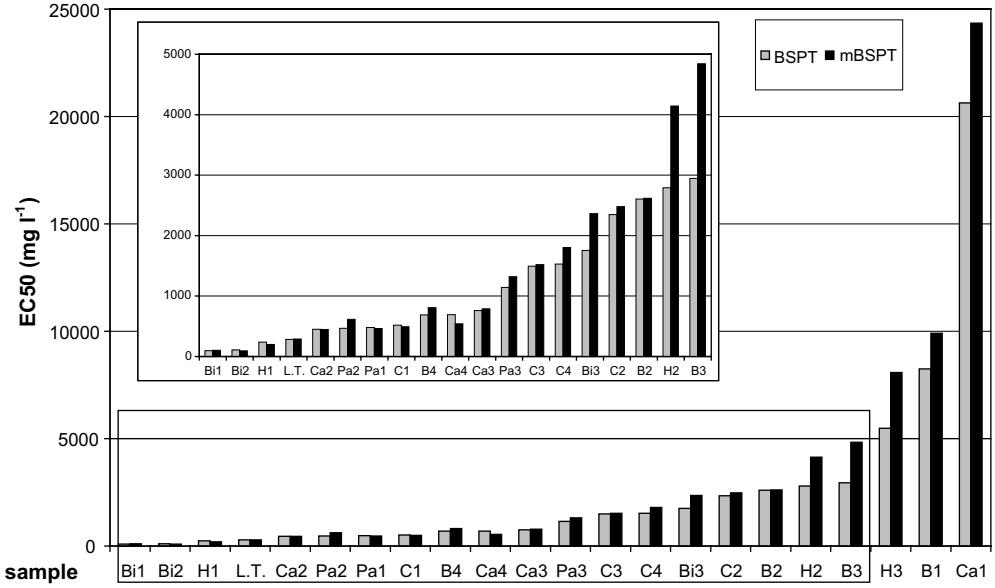

Fig. 2. Comparison between EC50 values, obtained with BSPT and mBSPT procedures, for all the test sample. For EC50 < 5000 mg l^{-1}, a zoom is provided.

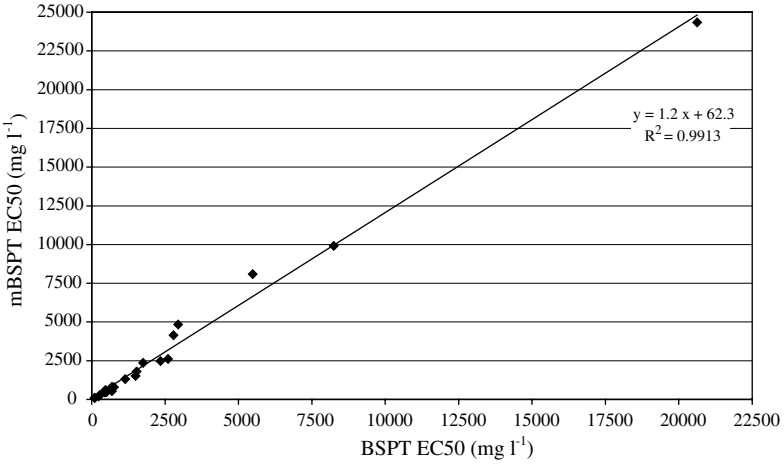

Fig. 3. Correlation of BSPT and mBSPT toxicity values (EC50) for all the sediment samples.

the dilution, produced by the addition of 500 µl sample in 510 µl bacteria reagent. When sediment suspensions at different initial concentration (from 0.48% to 27.5% dry weight) were dispensed, the sample turbidity and color produced a light reduction higher than 50%, resulting in a false inhibition. These results are in agreement with the findings of Lappalainen et al. (2001), where 0.5% caramel solution resulted in a 85% inhibition of light output, despite its untoxicity. Only using higher diluted sample suspension (for example sample Ca2, 0.05%), the sample slightly influenced light measurements. For sample Ca1 (reference sample), the light output decreased of 52% when sample suspension was added, increasing the sample toxicity of 18%. In any case, even thought mBSPT was carried out for sample Ca1, it was possible to record a reduction in light production (adverse effect), despite its not contamination. A recent study (Onorati and Mecozzi, 2004) demonstrated that the use of the SPT diluent for fresh marine sediments caused variations of pH,

Table 1
Effect of sample concentration on initial light output, before sample addition (I_0) and just after sample addition (I_{mod}), for 10 samples at different initial test concentrations

Sample	Concentration (%)	Signal		Change (%)
		I_0	I_{mod}	
Control (35‰ NaCl)	–	92 ± 18[a]	87 ± 16[a]	−5
Ca2	0.05	90	80	−11
H1	0.48	79	22	−73
Pa2	0.70	93	25	−73
Ca4	0.83	89	41	−54
Pa1	0.91	89	25	−72
H2	0.97	88	28	−68
B3	2.7	83	20	−76
H3	2.8	85	33	−61
Ca3	6.0	86	6	−93
Ca1 (not contaminated)	27.5	90	43	−52

[a] Mean of 22 data ± standard deviation.

which produced an additional stress factor for the bacteria, enhancing the risk of false-positive data. In this study, SPT diluent was used for all the tests, but no pH control was conducted during the tests. Seemingly, the adverse effect produced by sample Ca1 could have been influenced by the use of SPT diluent. Yet, anoxic unpolluted sediments can be toxic for aerobic microorganisms, as *V. fischeri* (Van Beelen, 2003).

As sample turbidity and color depend on particle fraction and humic substances, relationships between silt + clay fraction (<63 μm) and organic matter (OM) versus initial light loss were investigated. A poor correlation was found ($r^2 < 0.13$) in both cases. A better linear correlation ($r^2 = 0.41$, $p < 0.05$) was reached between initial light loss and the calculated amount of OM in the cuvette. For example, the organic matter content in sample H1 was twice than that in H2 and half sample concentration for H1 (0.48% instead of 0.97% for H2) was enough to produce the same light output inhibition recorded for sample H2 (−68%/−73%).

Influence of sample turbidity and color decreased at increasing test dilutions. In fact, the difference in initial light measurements, before and after sample addition, linearly increased with serial dilutions, depending on the sample quantity into the test cuvette, as shown in Fig. 4. To highlight this relationship, a selection of the most representative samples (Ca1, Pa3 and H1) was chosen on the basis of their toxicity (low, middle and high toxicity, respectively). For each sample concentration, the light output difference between BSPT and mBSPT (change %) was corrected with light output difference of test control (35‰ NaCl solution) and the absolute value was considered. A significant correlation ($r^2 > 0.91$, $p < 0.005$) was found for every sample, and in particular for the reference sample Ca1 ($r^2 = 0.98$). Anyway, the three linear regressions were different and seemed to be sample dependent.

In Fig. 5, a log–log regression between concentrations and light differences is reported. A significant correlation coefficient ($r^2 = 0.49$, $p < 0.005$) supports the hypothesis that when low dilutions (high concentrated sample suspension) were arranged in BSPT, in particular for less contaminated sample, they may cause incorrect readings, giving false toxic results.

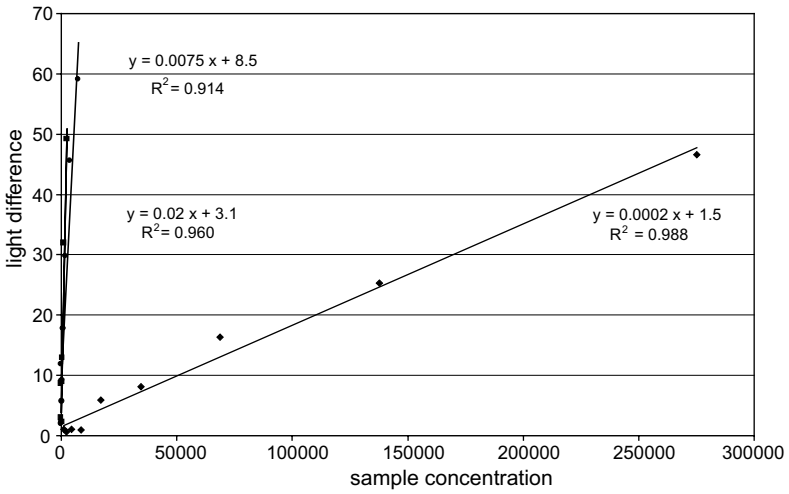

Fig. 4. Example of correlation between sample test concentrations and the light output difference, expressed as the absolute value of $[(I_0 - I_{mod})/I_0 \times 100]_{sample} - [(I_0 - I_{mod})/I_0 \times 100]_{control}$. Symbol (■) stands for sample H1, symbol (◆) stands for sample Ca1 and symbol (●) stands for sample Pa3.

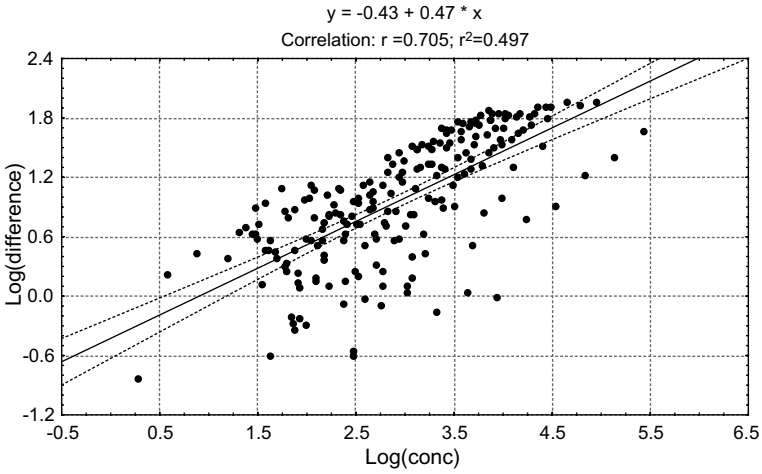

Fig. 5. Correlation between the sample test concentrations (log(conc)) and the light output difference (log(difference)), calculated as in Fig. 4.

4. Conclusions

This study indicated that the Basic Solid-Phase Microtox® assay (BSPT) could be affected by turbidity and color of sediments, and a significant bias can occur in the interpretation of the results. The influence of these confounding factors resulted in a significant decrease of bacteria light outputs due to physical effects during luminometer measurements, rather than real toxic effects. Therefore, the possibility that colored sediments would be identified as toxic, created the potential for high false-positives (Lappalainen et al., 1999). In order to overcome this problem, a simple and rapid modified procedure (mBSPT) was here suggested, without additional correcting methods, which could be time consuming. Measurements of sediments using mBSPT have been shown to be effective for investigating toxic effect and resulted not influenced by sample turbidity and color as it is the standard BSPT. The good correlation ($r^2 > 0.99$) between EC50 values for BSPT and EC50 values for mBSPT demonstrated that the differences in EC50 were recorded for all 22 samples. In addition, when applying the mBSPT procedure, the EC50 values resulted higher (less toxic) than those obtained with standard BSPT.

Regression studies showed that the correlation between sample concentrations and initial light output loss was very strong and sample dependent. This confirmed that the sample turbidity and color influenced the light output measurements, and the effect of the sediment particles on bacteria light emission masked the actual light emission, producing a signal drop higher than 50% and up to 93%. Conversely, poor correlations were found between light output loss and silt + clay fraction (<63 μm) or organic matter content. Thus, it was not possible to find a statistical relationship between bias light readings and particle fraction or organic matter content, as measures of sample turbidity and color.

To sum up, the toxicity values obtained with standard BSPT reflected sample actual toxicity, caused by sediment contaminants, and the interference on luminometer readings, due to sample concentrations. If the sample is slightly or non-toxic, then the sample turbidity or color should be the main contributor in toxicity measurement, producing false toxic results. This is in agreement with the findings on caramel solution in Lappalainen et al. (2001). With the new approach (mBSPT), the turbidity and color of the sample was taken into account during initial and final light measurements, considering sample effect at all time readings. More investigations should be conducted, in particular on non-contaminated samples, in order to confirm the effectiveness of modified Microtox® Basic Solid-Phase Test.

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