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Comparative evaluation of acute toxicity by *Vibrio fischeri* and fern spore based bioassays in the follow-up of toxic chemicals degradation by photocatalysis

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ABSTRACT

The development of efficient bioassays is a necessary step for cost-effective environmental monitoring and evaluation of novel decontamination technologies. Marine *Vibrio fischeri* kits have demonstrated to be extremely sensitive but lack of ecological relevance, especially when assessing impacts on freshwater higher organisms. A novel riparian are fern spore microbioassay could merge higher ecological relevance and reduced costs. The aim of this work is the comparative evaluation of the *V. fischeri* and fern spore bioassays for the follow up of detoxification processes of water contaminated with cyanide and phenol by advanced oxidation technologies, using heterogeneous photocatalysis as example. In both cases, EC_{50} values differed significantly for *V. fischeri* commercial kit, *V. fischeri* lab cultures and *Polystichum setiferum* fern spores (1.9, 16 and 101 mg cyanide L^{-1} and 27.0, 49.3 and 1440 mg phenol L^{-1}, respectively). Whereas *V. fischeri* bioassays are extremely sensitive and dilution series must be prepared, toxicant solutions can be directly applied to spores. Spore microbioassay was also useful in the follow up of photoxidation processes of cyanide and phenol, also reflecting the formation of intermediate degradation by-products even more toxic than phenol. We conclude that this new microbioassay is a promising cost-effective tool for the follow up of decontamination processes.

1. Introduction

Traditionally environmental risk monitoring of anthropogenic releases has been performed by means of chemical analysis. However, chemical analysis only provide a biased characterization of the sample since only a few targeted compounds are studied and the possible joint effects of by-products on the biota are neglected. In order to evaluate the real effect of hazardous materials on the environment or the efficacy of pollution control processes, analytical quantification of the parent and derived chemicals must be completed with toxicological studies on ecologically relevant organisms [1,2]. Thus, bioassays become necessary for the assessment of acute and chronic effects of hazardous chemical releases.

Widespread use of biological toxicity testing requires the availability of suitable methods. Traditional biological toxicity tests are usually long and also require specialized facilities able to host large numbers of specimen (copepods, fish, whole plants, etc.) in specific environmental conditions and where the handling and disposal of large volumes of treated matrix (i.e. water, soil, etc.) should be possible. Together with this, the large amounts of matrix to be treated increase the need for large volumes of the compound or environmental sample to be tested. These characteristics not only increase the test cost dramatically, but also can, in many cases, make the test unfeasible. Methodologies to reduce sample volume and space requirements have been attempted, from the traditional tests to more elaborated toxicity kits [2–6].

Environmental monitoring and assessment, therefore, faces at present the challenge to develop new cost-effective tools, more sensitive and reliable, with increased biological and ecological relevance than those currently used.

The use of toxicity bioassays is significantly important in the evaluation of the possible application of conventional biological processes in wastewater treatment plants [7]. Industrial wastewater usually presents significant amounts of highly toxic and non-biodegradable chemicals such as phenols or cyanides, that need to be removed before being sent to a conventional wastewater treatment plant, commonly through the application of advanced oxidation processes (AOPs) [8,9]. The usual goal of these processes is not the total mineralisation of the pollutants to substitute the cheaper conventional biological technologies, but to achieve a significant reduction of toxicity and an increase of biodegradability to make possible the coupling with a biotreatment. Consequently,

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the availability of reliable toxicity tests constitutes a key requirement for the proper evaluation and comparison of different AOPs [10–12].

Among the different toxicity bioassays reported in the literature, the inhibition of the photoluminescence of the marine bacterium *Vibrio fischeri* is by far the most widely chosen to follow the treatment of water containing phenol [8], pesticides [13], cyanides [14], drugs [15], etc. The main reason is that this test provides an easy way of getting quantitative toxicity data in short period of times, being the procedure supported by the ISO 11348 standard [16]. However, despite its popularity, the ecological relevance of the *V. fischeri* and the doubtful extrapolation of their toxicity results to conventional biotreatment sludge and/or higher organisms is frequently questioned.

Recently, a new bioassay of acute phytotoxicity during the development of fern spores has been published [4]. This higher plant testing method is naturally miniaturized and combines biological and ecological relevance together with sensitivity and simplicity. The versatility of this bioassay both for environmental [17] and toxicological studies [18] makes it a promising cost-effective tool for high throughput toxicity screening and monitoring. The aim of this work is the comparative evaluation of the *V. fischeri* and fern spore bioassays for the follow up of the photocatalytic degradation of cyanide and phenol, as an example of the application of advanced oxidation processes for the decontamination of water with toxic and non-biodegradable chemical pollutants. Heterogeneous photocatalysis using TiO_2 as catalyst has been long studied due to the advantages over other advanced oxidation processes (AOPs), such as operation under ambient temperature and pressure, no requirements of pH adjustment or additional oxidant chemicals apart from air, and also the possibility of using solar light as radiation source. This process is based on the use of a semiconductor material that generates electron/hole pairs that eventually lead to the formation of oxidant and reductant secondary species such as superoxide anions and hydroxyl radicals when being illuminated with radiation above the energy of its band-gap.

2. Experimental

2.1. Bacterial luminescence bioassay

This is one of the most widely employed methods in wastewater and environmental monitoring, and it is based on the decay of the light emitted by *V. fischeri* when exposed to toxic chemical compounds. The light emission of luminescent bacteria is produced by the oxidation of a reduced flavin mononucleotide ($FMNH_2$) catalysed by the mixed-function oxidase known as luciferase which is concomitant with the oxidation of a long chain aliphatic aldehyde. This reaction renders light together with an oxidized mononucleotide (FMN) and a fatty acid which are reduced again to the initial compounds with a high energy cost. Luciferase activity can be reduced by several causes: direct inhibition of the enzyme by the toxicant, depletion of the cell's reducing power (NADH) for FMN recycling or reduction of the bacterial density (i.e. death) [19]. Since a decrease in light emission is, in any case, the response to a serious damage to bacteria, the percentage of luminescence inhibition is used as a measure for toxicity.

2.2. V. fischeri commercial kit bioassay

A *V. fischeri* toxicity BioTox™ kit supplied by Gomensoro S.A. was used to carry out the *V. fischeri* bioassay. The lyophilised bacteria were stored at −18 °C. For their resuspension, 2 mg of the bacteria were rehydrated in 2 mL of a 2% NaCl solution and then, incubated at 15 °C in a thermostatically controlled block during 1 h in order to rule out any decay in the bacterial luminescence as a consequence of the natural bacterial death due to their adaptation to a new medium.

2.3. V. fischeri laboratory culture bioassay

Bacterial suspensions were prepared from lyophilised *V. fischeri* provided by the Colección Española de Cultivos Tipo (CECT 524, corresponding to ATCC 7744). Lyophilised bacteria were resuspended in 0.3 mL of sterilized nutrient medium (Marine Broth, Scharlab) and then, added to 20 mL of this sterilized marine nutrient medium. Finally, this bacterial liquid culture was incubated at 24 °C for 24 h under a 60 rpm constant stirring in a thermostatically controlled water bath. This initial liquid culture was kept at 4 °C in dark. Fresh liquid cultures were weekly prepared by inoculation of 200 μL of the initial bacterial liquid culture in 20 mL of sterilized marine nutrient medium and incubation at 24 °C for 24 h under a 60 rpm constant stirring in a thermostatically controlled water bath. Finally, a specific volume was taken and diluted with a 2% NaCl solution up to 2×10^3–1×10^6 RLU (Relative Light Units), measured in a Luminometer Optocomp I, MGM Instruments, INC.

For both *V. fischeri* bioassays, toxicological parameters of both, cyanide and phenol, such as EC_{50} (concentration which produces a 50% reduction in bioluminescence in the marine bacterium *V. fischeri* in comparison with that of a control) were evaluated according to the standard ISO 11348-3. Three kinds of tests were carried out. Firstly, a control of bacteria bioluminescence was required to choose samples showing a similar bioluminescence (samples with differences in light emission of 10% were ruled out). Secondly, a screening test was done to measure the bioluminescence inhibition in presence of cyanide or phenol. Stock solutions of 100 mg L^{-1} of cyanide and 1000 mg L^{-1} of phenol were prepared in a 2% NaCl solution and subsequently, adjusted to a pH value of 7. Finally, a range of different dilutions were prepared from the stock solutions in a 2% NaCl solution to measure the reduction in intensity of light emitted from the bacteria for different concentrations of cyanide and phenol. For the ecotoxicity test, the bioluminescence inhibition of the bacteria was measured along these dilutions, a blank (200 μL of a 2% NaCl solution) and a control (100 μL of bacteria and 100 μL of a 2% NaCl solution). A 4 mg L^{-1} $K_2Cr_2O_7$ solution was also used as a reference chemical compound since it is known the percentage of bioluminescence inhibition produced by this compound after 30 min of exposure. Then, 100 μL of bacteria previously incubated at 15 °C for 15 min and also kept at 15 °C during all the test in a thermostatically controlled block were distributed to test tubes. An initial light output (I_0) from each test tube was recorded, accepting 10% as a maximum value of average deviation. This was followed by the addition of 100 μL of each dilution also incubated at 15 °C to the appropriate test tubes. After a 15 min exposure period, the final light output (I_{15}) was measured. The change in light output corresponds to the light inhibition percentage and it was determined according to the standard ISO 11348-3. The concentration of the toxic produces a dose–response relationship and the result is presented as the 50% inhibition effective concentration (EC_{50}).

Concerning the samples taken through the decontamination process of effluents containing cyanide or phenol, their ecotoxicity was also analysed by the screening test, based on the measure of the percentage of bioluminescence inhibition of the marine bacterium *V. fischeri*, according to the standard ISO 11348-3. The value of pH of the samples was adjusted to 7 before the analysis.

2.4. Fern spore bioassay

Polystichum setiferum spores were sampled in NW Spain, A Coruña province, San Xusto river. Fragments of leaf were collected

with mature but closed sporangia. Spore release was promoted by drying the fragments on smooth paper for a week in the laboratory. Dry spores were stored at 4 °C in darkness until use.

The fern spore bioassay was performed as described in detail elsewhere [17] with minor modifications. Briefly, aliquots containing 8×10^4 spores were prepared and 1.5 mL of each sample was added. Control treatment samples contained 1.5 mL of Dyer medium supplemented with Tween 20, 0.005%. Spores were incubated with the pH neutralized reaction product during 20 h in controlled conditions (20 °C, PAR 35 μmol m^{-2} s^{-1} 16 h photoperiod). Immediately afterwards mitochondrial activity was assessed with 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany) 0.6%, pH 8 during 2 h in darkness. The homogenisation of the spores was performed in several steps: first, a mixture consisting of 240 μL KOH 0.1 N and 240 μL ethanol 96% (v/v) was added to the spore pellet and sonicated in a Selecta Ultrasons bath for 1 h (40 kHz, 100 W) at room temperature, then samples were incubated at 65 °C during 15 min in a water bath. Finally, approximately 200 μL of sieved glass fragments (0.2–1 mm) were added to the tubes prior to 30 min of agitation (3000 rpm, Labnet, Edison, NJ, USA) to achieve a completely homogeneous sample. The coloured formazan salt, produced by the reduction of TTC in mitochondria, was extracted in 1000 μL of n-hexane and formazan absorbance was read at 492 nm in a Spectronic Genesys 8 UV/vis spectrophotometer. Mitochondrial activity of spores solely exposed to culture medium was assessed as a control treatment.

2.5. Photocatalytic degradation reactions

The experimental setup consists of an annular photoreactor of 1250 cm^3 irradiated volume, operating in a closed recirculating circuit with a reservoir tank of 4 L of total working volume driven by a centrifugal pump at a flow rate of 65 cm^3 s^{-1}. Illumination was carried out using an Osram L 36 W black light lamp with a maximum emission peak centred at 365 nm placed in the axis of the reactor. The UV-A incident photon flow determined by ferrioxalate actinometry was 1.8×10^{-5} Einstein s^{-1}. A scheme of the reactor and more details of its dimensions and characteristics can be found elsewhere [20].

Degradation experiments have been carried out using a catalyst concentration of 0.5 g L^{-1} of Degussa P25 TiO_2, a well-known semiconductor usually considered as reference materials for photocatalytic experiments. It consists of a mixture of 70% anatase and 30% rutile crystalline phases and presents a band-gap energy corresponding to a UV-A absorption band below 390 nm. This optimal catalyst concentration of 0.5 g L^{-1} has been selected after preliminary experiments.

Reacting solutions were prepared by dissolving the selected toxic chemicals (potassium cyanide, Panreac; phenol, Sigma–Aldrich) in deionised water (Milli-Q®, 18.2 MΩ cm). In the case of cyanide degradation experiments, an initial concentration of 400 mg L^{-1} of CN^- was prepared, and the pH was adjusted to 12 with sodium hydroxide (Scharlab) to avoid evolution of gaseous HCN. Samples were taken from the reservoir tank to follow the reaction evolution. After measuring temperature and pH to check that both parameters remained essentially constant, the catalyst was removed from the sample by filtering the suspension through 0.22 μm nylon membranes. Cyanide analysis was carried out using the pyridine–barbituric standard colorimetric method [21]. Experiments of phenol degradation were carried out starting from a 1000 mg L^{-1} initial concentration. Phenol conversion was monitored with a HPLC chromatograph Varian Prostar equipped with a Waters Spherisorb column (250 mm × 4.6 mm) and an UV detector at 215 nm. Phenol and other by-products coming from its incomplete mineralisation were separated using deionised water (Milli-Q®, 18.2 MΩ cm^{-1}) acidified with 1 mL L^{-1} of orthophosphoric acid as mobile phase at a flow rate of 0.5 mL min^{-1}.

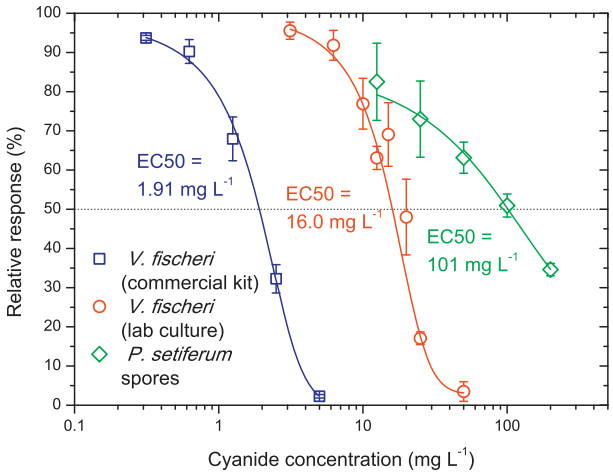

Fig. 1. Effect of cyanide concentration on the inhibition of *V. fischeri* bioluminiscence and *P. setiferum* mitochondrial activity. EC_{50} values have been calculated by fitting the experimental data to dose–response curves with variable Hill slope.

The value of pH of the samples obtained from the reactor was always adjusted to 7 before performing the toxicity tests.

2.6. Statistics

Differences among bioassay measurements were assessed with ANOVA and a post hoc Tukey-b test. A Levene test for homogeneity of variance was performed to verify data normality. Statistical significance was established at $p < 0.05$. All bioassays were performed at least three times and at least in duplicate per concentration.

3. Results and discussion

3.1. Toxicological study

3.1.1. Cyanide

Fig. 1 shows the comparison between the dose–response curves for the inhibition of the response of the studied bioassays for increasing concentrations of cyanide. As it can be seen, the value of the half-maximum inhibition effective concentration (EC_{50}) is significantly different for each bioassay, from 1.9 mg L^{-1} for the *V. fischeri* commercial kit (in agreement with the EC_{50} value of 1.8 mg L^{-1} reported [22]), to 16 mg L^{-1} for the *V. fischeri* lab culture and 101 mg L^{-1} for the *P. setiferum* bioassay. These results were partially expected, as the acute phytotoxicity determined for higher plants is usually lower than the ecotoxicity values obtained using very sensitive marine bacteria such as *V. fischeri*. Not so expected was the comparison between the almost ten times higher value of EC_{50} calculated by using lab cultures of *V. fischeri* in comparison with commercial lyophilised kits. The toxic mechanism of cyanide primarily involves the inhibition of cytochrome electron carriers of the electron transport chain, restraining the cellular respiration. Consequently, a plausible explanation for this result is that the lab cultures, collected for the assays during the early stationary phase of the growing curve, are in a more favourable physiological state to overcome the toxic stress than the freshly reconstituted and rehydrated commercial kit bacteria. Freeze injury in bacteria may especially affect membrane and cell wall integrity and toxicants might leak into the cell through damaged areas. This effect must not be neglected since reconstituted bacteria must face the combined stress of freeze injury repair and a magnified toxicant insult (reviewed in [23]). This high sensitivity of the experimental results

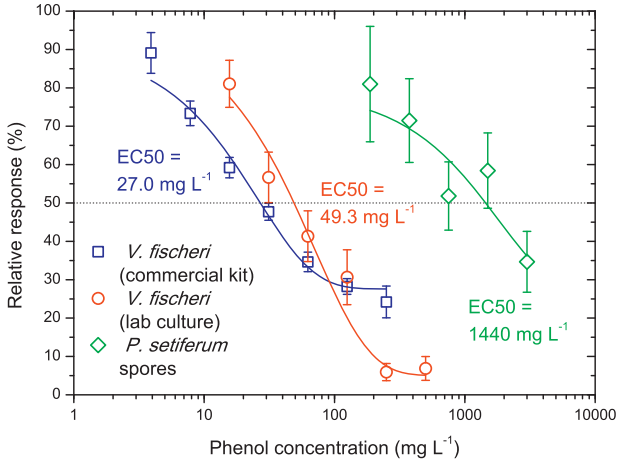

Fig. 2. Effect of phenol concentration on the inhibition of *V. fischeri* bioluminiscence and *P. setiferum* mitochondrial activity. EC_{50} values have been calculated by fitting the experimental data to dose–response curves with variable Hill slope.

to the biological state of the bacterial suspensions also explained the relatively high dispersion of values of EC_{50} usually reported in the literature.

Instead of being a drawback, the discrepancy between the values of EC_{50} provided for these three different bioassays constitute a combination of tools with different sensitivity for the evaluation of the acute toxicity of waters over a wide range of concentrations of toxic compounds. For instance, the use of the *V. fischeri* commercial kit provided a very sensitive bioassay for the evaluation of the toxicity of low toxic environmental samples or diluted wastewater, whereas the lower sensitivity of the *P. setiferum* spores bioassay would allow the direct determination of the toxicity of contaminated water or industrial wastewater with much higher concentrations in toxic chemicals that would lead to a total inhibition of the response of the *V. fischeri* bioassays even after dilution.

3.1.2. Phenol

Fig. 2 shows the comparison between the dose–response curves for the inhibition of the response of the studied bioassays for increasing concentrations of phenol. Again, the EC_{50} value of 1440 mg L^{-1} calculated for the *P. setiferum* spores assay is two orders of magnitude higher than that calculated using the *V. fischeri* commercial kit (27 mg L^{-1}, in agreement with the EC_{50} value of 23.5 mg L^{-1} reported [7]). However, in this case, the EC_{50} value calculated using *V. fischeri* lab cultures is only twice instead of ten times higher than that of the commercial kit, as it happens for cyanide. The reason of this different behaviour could be the more general toxic mechanism of phenol by induction of cell oxidative stress that can be compensated by antioxidant defence mechanisms, what makes the response less sensitive to the physiological state of the bacteria than the respiratory specific toxic mechanism of cyanide.

As it happens with cyanide, the toxicological results of phenol confirm that the combination of different bioassays provides a sensitive tool for the evaluation of the acute toxicity of waters over a wide range of toxic concentrations.

3.2. Toxicological monitoring of AOPs

3.2.1. Cyanide degradation

Photocatalytic experiments of cyanide oxidation show that a complete degradation following a pseudo-first order kinetics can be achieved, even for high concentrations above 400 mg L^{-1}. A closed mass balance is found between the disappearance of cyanide and the formation of cyanate (not shown) in agreement with previous

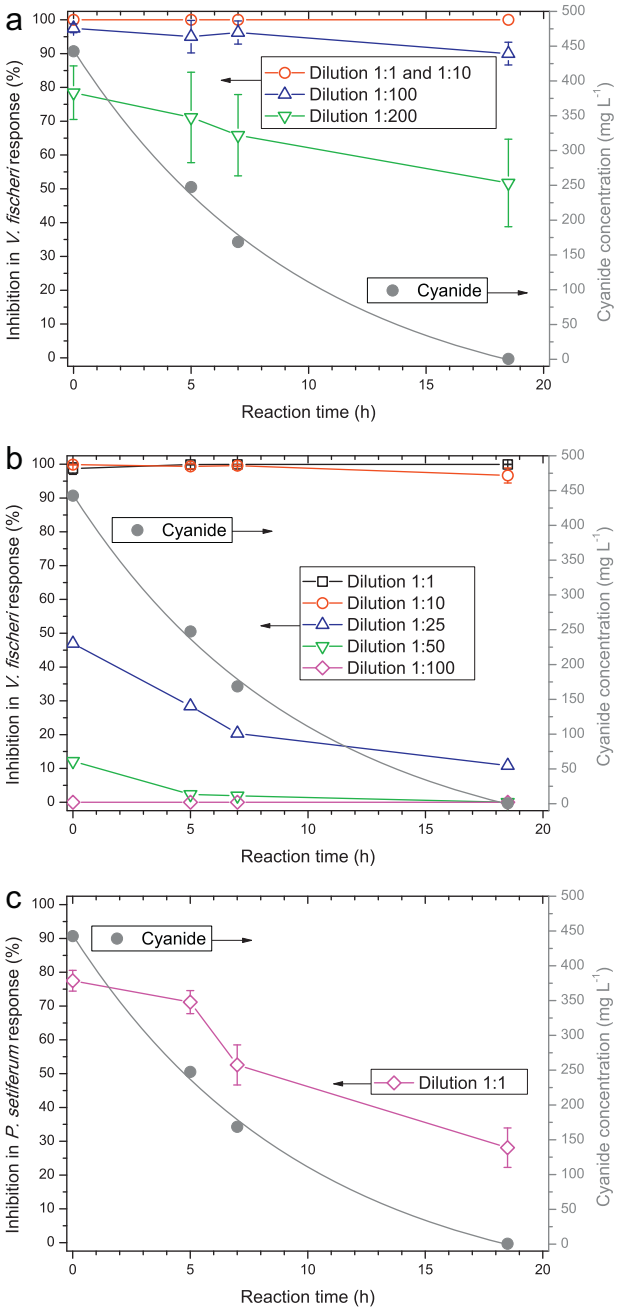

Fig. 3. Evolution of toxicity during cyanide degradation reaction: (a) inhibition of *V. fischeri* using the commercial kit; (b) inhibition of *V. fischeri* using the lab culture; and (c) inhibition of *P. setiferum* mitochondrial activity.

studies [24]. Fig. 3 shows the relation between the disappearance of cyanide and the evolution of the inhibition of the response of the three bioassays. In all cases, a clear decrease in the toxicity is observed, as a result of the progressive transformation of cyanide into the less toxic cyanate. However, significant differences can be observed in the performance of each toxicological test. The use of *P. setiferum* fern spores bioassay allows the direct follow up of the process without dilution of the water samples, with an evolution from nearly 80% of inhibition at the beginning of the reaction to a value below 30% at the point in which cyanide has completely disappeared. In contrast, both *V. fischeri* bioassays require an important dilution of the samples before being tested, because otherwise a total inhibition would be always observed. In the case of the *V. fischeri* commercial kit, the sensitivity in the inhibition response is

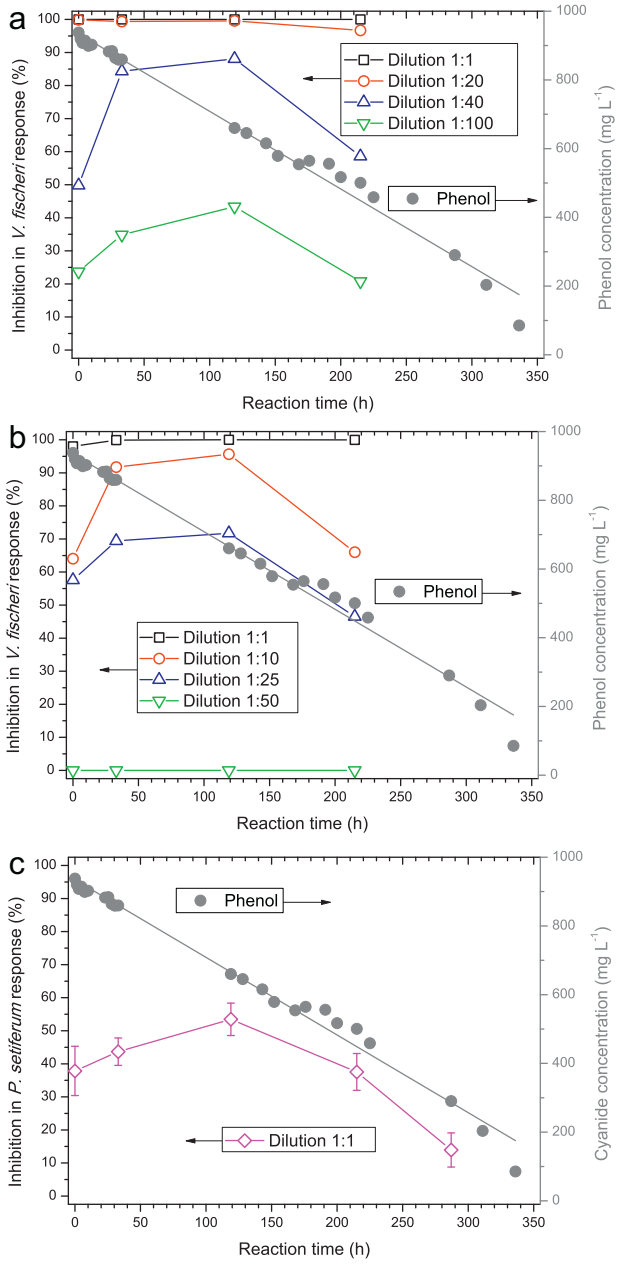

Fig. 4. Evolution of toxicity during phenol degradation reaction: (a) inhibition of *V. fischeri* using the commercial kit; (b) inhibition of *V. fischeri* using the lab culture; and (c) inhibition of *P. setiferum* mitochondrial activity.

only found after dilutions above 1:200, whereas the use of the lab cultures provides a sensitive response for dilutions around 1:25. Small dilutions lead to a total inhibition of the bioluminescence signal, whereas large dilutions lead to a negligible inhibition. Therefore, it can be concluded that whereas the use of *V. fischeri* bioassays is more sensitive to low concentrations of toxics, the possibility of direct application of *P. setiferum* bioassay makes it a more suitable option for the evaluation of processes for the treatment of highly contaminated water. *P. setiferum* is a widespread riverbank species of the temperate region which adds ecological relevance to the bioassay results, especially regarding the impact of discharges into surface waters.

3.2.2. Phenol degradation

As shown by a large number of literature reports, phenol can be also completely degraded by advanced oxidation processes,

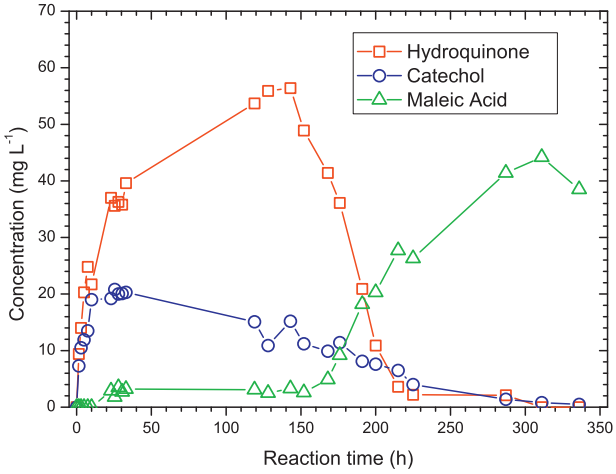

Fig. 5. Evolution of selected intermediate species during phenol degradation reaction.

including heterogeneous photocatalysis. Fig. 4 shows the evolution of the inhibition of the response of the three bioassays together with the phenol degradation profile. In this case, a different toxicity pattern is observed in comparison with the results of cyanide degradation previously shown, with an initial increase in the response inhibition at the beginning of the reaction followed by a maximum and then a decrease in the toxicity response to values below the initial one. This behaviour has been previously reported in the literature [25] in the monitoring of phenol wet catalytic oxidation by using *V. fischeri* toxicity measurements, and attributed to the formation of intermediate degradation by-products even more toxic than phenol. This hypothesis is supported by the profiles of formation and degradation of selected intermediate oxidation by-products displayed in Fig. 5. As it can be seen, the concentration of species derived from the partial oxidation of phenol such as hydroquinone and catechol clearly correlates the toxicity profiles, starting the net decrease of the toxicity response only after the almost complete disappearance of the aromatic intermediates.

Concerning the comparison of the different bioassays, *P. setiferum* fern spores toxicity test also reproduces this maximum in the inhibition correlated with the formation of aromatic intermediates more toxic than phenol, corroborating the validity of this novel bioassay for the follow up of decontamination processes. As it happens for cyanide, once again significant differences can be observed in the performance of each toxicological test. Whereas *P. setiferum* bioassay allows the direct follow up of the process without dilution, both *V. fischeri* bioassays require an important and very precise range of dilution of the water samples to achieve the sensitivity of the response to the treatment.

4. Conclusions

V. fischeri suspensions from lyophilised commercial kits show an exacerbated sensitivity to toxicants due to reconstitution stress. The use of lab cultures provides less sensitive but more realistic results. The recently developed toxicity bioassay using *P. setiferum* fern spores has been shown to be really useful for the direct evaluation of decontamination processes such as advanced oxidation processes, in which the analysis of individual chemical species would fail in assessing the environmental risk of the effluent. This method combines the advantages of simplicity and possibility of automation of miniaturised methods such as *V. fischeri* bioluminescence bioassay, relevant aspects from the economical viewpoint, with a higher biological and ecological relevance due to the use of a higher organism, and the possibility of direct use with highly

toxic water without requiring dilution. Therefore, this new bioassay, either alone or in combination with other toxicity bioassay, is presented as a promising tool for the follow up of processes for decontamination of industrial wastewater.

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