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Confirming *Pseudomonas putida* as a reliable bioassay for demonstrating biocompatibility enhancement by solar photo-oxidative processes of a biorecalcitrant effluent

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ABSTRACT

Experiments based on *Vibrio fischeri*, activated sludge and *Pseudomonas putida* have been employed to check variation in the biocompatibility of an aqueous solution of a commercial pesticide, along solar photo-oxidative process (TiO_2 and Fenton reagent). Activated sludge-based experiments have demonstrated a complete detoxification of the solution, although important toxicity is still detected according to the more sensitive *V. fischeri* assays. In parallel, the biodegradability of organic matter is strongly enhanced, with BOD_5/COD ratio above 0.8. Bioassays run with *P. putida* have given similar trends, remarking the convenience of using *P. putida* culture as a reliable and reproducible method for assessing both toxicity and biodegradability, as a substitute to other more time consuming methods.
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1. Introduction

Increasing amounts of pesticides are employed in intensive agriculture, making water polluted with these chemicals a related environmental concern. These effluents are not compatible with classical biological treatment, due to the toxicity shown towards micro-organisms involved in those processes [1]. For this reason, much effort has been devoted to develop alternative methods to deal with this problem.

One promising strategy is to employ a chemical pre-treatment to improve the biocompatibility of the effluent, followed by an activated sludge-based biological reactor [2,3]. In this context, photocatalysis has proven to be a useful method to remove toxic pollutants from aqueous effluents; in some cases, sunlight can be used as irradiation source, enhancing the sustainability of the process [4]. In particular, TiO_2-based photocatalysis and the photo-Fenton reaction (catalytic iron salts and sacrificial hydrogen peroxide as oxidant) can be driven under solar irradiation; different pesticides have been treated by these methods and primary elimination of these chemicals has been accomplished [5,6].

There are some studies indicating that solar-driven photo-oxidative pre-treatments are able to detoxify pesticide containing solutions. Different bioassays have been used to assess detoxification, among them the highly sensitive inhibition of the luminescence of the *Vibrio fischeri* bacteria [7], or the inhibition of the respiration of activated sludge, which is expected to be more closely related with the behaviour of biological reactors [8]. Furthermore, an increase in the biodegradability of the sample has been also measured employing either the biological oxygen demand [9] or the Zahn–Wellens test [10]. Finally, some experiments have been reported in which photocatalytic reactors have been coupled with activated sludge-based reactors [11].

An alternative assay to determine the biocompatibility of the treated effluent is to study the growth of a selected micro-organism in the presence of the pesticide solution after different periods of photochemical pre-treatment. The use of *Pseudomonas putida* for this purpose seems interesting as it is commonly a part of the

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consortium of micro-organisms present in activated sludge; these assays might combine the improved reproducibility of using a more simple system than activated sludge, with a behaviour that is not expected to differ substantially to that of the biological reactors [12]. Furthermore, biodegradability assay using *P. putida* is faster (some few days) than the widely used Zahn–Wellens test for which 28 days are needed.

In this paper, experiments based on *V. fischeri*, activated sludge and *P. putida* have been employed to check variation in the biocompatibility of an aqueous solution of a commercial pesticide, along solar photo-oxidative process. Sevnol, a commercial product containing carbaryl as active ingredient has been chosen as target pollutant, as previous works have demonstrated that it can be removed by photochemical means [13–15], although a significant amount of organic matter remained in the solution.

2. Experimental

2.1. Reagents and micro-organisms

Commercial Sevnol (85% (w/w) of active ingredient, carbaryl) was purchased from MAFA and used as received. Titanium dioxide (Degussa P-25) was employed as heterogeneous photocatalyst. Hydrogen peroxide (30%, v/v) and ferrous sulphate employed in the photo-Fenton reaction were purchased from Panreac and used without further purification.

Biological assays were performed using activated sludge taken from the exit of the biological reactor of the wastewater treatment plant from Alcoy (Spain). The sludge was diluted to keep the amount of suspended solids in the range 1–2 $g\,L^{-1}$; it was employed without previous adaptation to the pollutants, in order to reproduce more closely the behaviour of the plant.

P. putida CECT 324 was acquired from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, Valencia, Spain). Cultures were grown at pH 7.2 in beef extract, 1 $g\,L^{-1}$, yeast extract, 2 $g\,L^{-1}$, peptone, 5 $g\,L^{-1}$, NaCl, 5 $g\,L^{-1}$ and agar powder, 15 $g\,L^{-1}$, and maintained at $-70\,^{\circ}C$ in a cryogenic solution (glycerol 87%).

2.2. Photocatalytic reactions

Solar photodegradation of the pesticide was performed in a pilot plant for wastewater detoxification (Solardetox Acadus 2001, Ecosystem), based on compound parabolic collectors, CPCs, able to treat up to 25 L of wastewater with 2.0 m^2 irradiated surface and 15.1 L irradiated volume [16]. Accumulated and instantaneous UV-radiation could be measured by a radiometer (Acadus 85). The $t_{30\,W}$ parameter was employed to normalise solar irradiation: first the accumulated UV-radiation received by the plant is measured with a radiometer, and then it is converted into a time scale ($t_{30\,W}$) by considering a standard intensity of 30 $W\,m^{-2}$ for sunlight [17], which is a typical UV-radiation on a sunny day around noon in the South East of Spain. Eq. (1) is used for these calculations, where UV_{ac} is the accumulated solar UV-radiation ($J\,m^{-2}$), V_i the irradiated volume and V_t the total volume:

$$t_{30W} = \frac{UV_{ac}}{30}\frac{V_i}{V_t} \quad (1)$$

For the reactions driven in the presence of TiO_2, the initial concentration of carbaryl was 50 $mg\,L^{-1}$ and the amount of photocatalyst was 0.5 $g\,L^{-1}$. In the photo-Fenton reaction, the initial concentration of carbaryl was close to saturation, 136 $mg\,L^{-1}$. The pH was adjusted to 2.8 with sulphuric acid, the concentration of Fe^{2+} was 20 $mg\,L^{-1}$ and the hydrogen peroxide was continuously added throughout the process, to keep its concentration in the range of 200–500 $mg\,L^{-1}$. Samples were filtered through polypropylene before chemical or biological analysis. In the case of the photo-Fenton process, absence of residual hydrogen peroxide in the samples was checked.

2.3. Chemical analyses

Carbaryl concentration was determined by means of HPLC (PerkinElmer XL Autosystem, equipped with a diode-array detector and an autosampler). A reverse phase column (LiChrosphere 100 RP-18) was employed, with a mixture of methanol (52%) and H_2SO_4 0.01 M (48%) as eluent (1 $mL\,min^{-1}$ flow). Detection was based on the absorption at 280 nm. Samples were diluted with methanol (1:1) before filtration.

Dissolved organic carbon (DOC) was determined with a Shimadzu model TOC-V CSH apparatus, based on combustion/non-dispersive infrared gas analysis method. Chemical oxygen demand (COD) determination was carried out spectrometrically, according to the dichromate standard method [18]; all reagents employed in this analysis were supplied by Merck. The concentration of hydrogen peroxide was determined by reaction with potassium iodide and posterior titration with sodium thiosulphate.

2.4. Activated sludge and V. fischeri-based biological assays

Inhibition of the oxygen uptake rate (OUR) experiments, based on the OECD 209 test, were carried out using an activated sludge respirometer BM3-LAB (Neurtek) equipped with an oxygen sensor (WTW-Cell Ox). OUR was obtained from the difference in the concentration of oxygen in the activated sludge when pumped to the oxygen sensor through two pathways of different length [19]. Briefly, activated sludge (500 mL) was brought to its maximum oxygen uptake rate (OUR_{max}) by addition of 1 g of solid sodium acetate. Then, 250 mL of the aqueous solution of Sevnol were added and the final oxygen consumption (OUR_f) was determined. The inhibition was calculated by Eq. (2a). Dilution of the sludge by addition of the liquid sample was responsible of some decrease in the OUR, as determined by blank experiments (I_B); thus, a corrected inhibition was calculated by means of Eq. (2b):

$$I(\%) = \frac{OUR_{max} - OUR_f}{OUR_{max}} \times 100 \quad (2a)$$

$$I_c(\%) = \frac{I - I_B}{100 - I_B} \times 100 \quad (2b)$$

BOD_5 determinations were carried out according to the standardised manometric method (OECD-301 series), using an OxiTop©(WTW) to seal the bottle, which contained a pressure-sensitive material which is able to measure the depression inside.

For the inhibition experiments based on the BOD_5, the manometric method was also employed. The same procedure described above for BOD_5 determination was followed, but in this case a highly biodegradable mixture of glucose (150 mg) and glutamate (150 mg) were added to the pesticide solution (BOD_{5inh}). In a blank experiment, glucose and glutamate were added to a distilled water solution and the BOD_5 was also calculated (BOD_{5B}). The inhibition was calculated according to the following equation:

$$\text{inhibition}\,(\%) = \frac{BOD_{5B} - BOD_{5inh}}{BOD_{5B}} \times 100 \quad (3)$$

Luminescent assays were performed according to the standardised ISO 11348-3 norm, using lyophilised *V. fischeri* bacteria, which was purchased from Macherey–Nagel as a commercial kit (NRRL B-11177). Luminescence was measured by a Luminometer Lumifix-Bio-10, also supplied by Macherey–Nagel. Toxicity was determined after 15 min incubation.

2.5. *P. putida* cultures

For biodegradability and toxicity studies for carbaryl, duplicated cultures were incubated at 30 °C on a rotary platform shaker (150 rpm, 2.6 cm stroke) in a 100-mL Erlenmeyer flask filled with 20 mL of different solutions (0–140 mg L^{-1}) of carbaryl. The flasks were inoculated with 80 μL of bacterial stock described above, previously melted at room temperature.

Photo-treated solutions of the pesticide were also submitted to biodegradability studies. Duplicated cultures were incubated at 30 °C on a rotary platform in a 250-mL Erlenmeyer flask filled with 50 mL of culture medium. The flasks were inoculated with 200 μL of bacterial stock at −70 °C. Carbaryl solutions (136 mg L^{-1}) at different treatment times constituted an additional carbon source. Samples were filtered through 0.20-μm syringe filters (AAWG, 47 mm, Millipore). The pH value was adjusted at 7.0 with NaOH 0.1N addition. Inoculums' viability was checked with 80 μL of bacterial stock in 100-L Erlenmeyer flasks filled with 20 mL of culture medium described in Section 2.1.

The baseline medium in all flask cultures (beef extract, 3.2 mg L^{-1}, yeast extract, 6.4 mg L^{-1}, peptone, 16 mg L^{-1}, NaCl, 16 mg L^{-1} glycerol 87% (v/v), 0.8 mL L^{-1}, 0.5 g L^{-1} NH_4Cl, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 10 mL L^{-1} of trace mineral solution) provides a DOC concentration of 360 mg L^{-1} approximately and is considered as a biodegradable carbon source. Initial bacterial concentration was 20 mg L^{-1} in all flask cultures.

3. Results and discussion

3.1. Toxicity and biodegradability test

Solar photodegradation of Sevnol carried out in the presence of titanium dioxide showed that complete removal of the active ingredient was achieved at a normalised irradiation time of 150 min (Fig. 1). When primary degradation of carbaryl was complete, the percentage of mineralization was only around 50% (DOC/DOC_0 = 0.5). This is in agreement with the literature, mineralization could not be achieved in this experiment, due to the generation of organic intermediates, which are not easily photo-oxidised to CO_2. However, trends in the COD indicate that there is a strong oxidation of the organic matter.

This oxidation is shown more clearly by the average oxidation state (AOS), which was calculated according to Eq. (4), where COD is expressed as mmol(O_2) L^{-1} and DOC as mmol(C) L^{-1}. AOS takes values between +4 for CO_2, the most oxidised state of C, and −4 for CH_4, the most reduced state of C. As observed in Fig. 1, the maximum

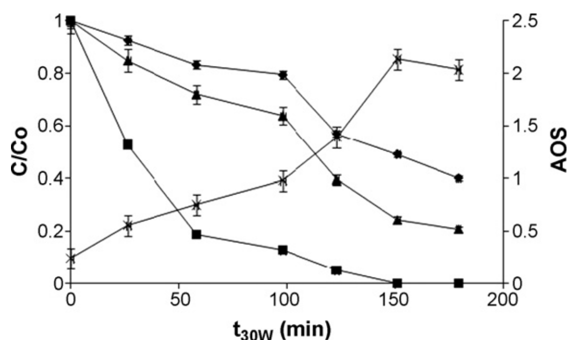

Fig. 1. Photodegradation of Sevnol catalysed by TiO_2 under solar irradiation. Left *Y*-axis, given as the ratios between these parameters and their initial values: (■) concentration of carbaryl, (▲) COD and (♦) DOC. Right *Y*-axis, (×) average oxidation state (AOS). The initial values for carbaryl concentration (C_0) was 34 mg L^{-1}, TOC_0 was 37 mg L^{-1} and COD_0 was 86 mg L^{-1}.

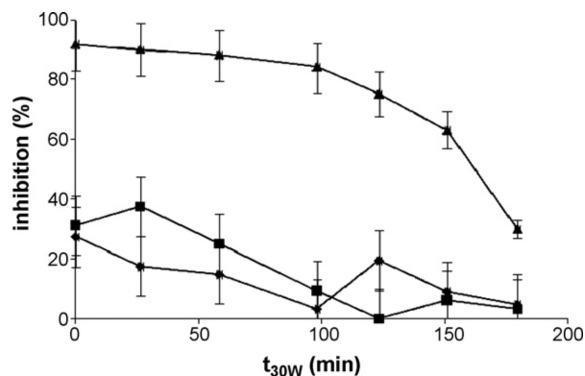

Fig. 2. Estimation of the toxicity for a solution of Sevnol after different periods of solar irradiation in the presence of TiO_2 according to different methods: (♦) corrected inhibition of the OUR of activated sludge, (■) inhibition of the BOD_5 and (▲) inhibition of the luminescence of *V. fischeri*.

AOS was reached at the end of the treatment after approximately 180 min of illumination time. AOS usually increased with treatment time until almost reaching a plateau [20], which was not observed in this case and it was probably due to the fact that the experiment was not long enough. The AOS increases from +0.2 at the beginning of the experiment to reach +2.4, which indicates that important changes towards more oxidised photoproducts in the composition of the dissolved organic matter occurred, and consequently, changes in its biocompatibility could be expected. Formation of more oxidised intermediates indirectly demonstrates that the treatment could improve biodegradability:

$$AOS = \frac{4(DOC - COD)}{DOC} \quad (4)$$

Inhibition of the respiration of activated sludge was employed to determine the toxicity of the treated solution. This is a fast response method which estimates the acute toxicity by a sharp decrease of the respiration rate upon addition of the toxicant. Fig. 2 shows a plot of the corrected inhibition of the OUR vs. the t_{30W}; this indicates that an important detoxification was achieved during the photocatalytic process as values around 25% were measured for the untreated solution, while the inhibition was negligible at the end of the experiment.

Although the inhibition of the OUR is a fast and convenient test for toxicity, the possible cumulative effect of the pollutants towards the micro-organisms present in the activated sludge cannot be detected and a more time consuming assay would be needed for this purpose. In this context, the inhibition of the activity of the activated sludge was estimated by the change of the BOD_5 of a very biodegradable mixture of glucose/glutamate in the presence of the toxicant [8]. In this case, the inoculum is submitted to the toxicant for 5 days, and thus the possible long-time effect of the chemicals is likely to be detected. Data in Fig. 2 indicate that inhibition calculated according to this method followed similar trends to OUR-based with an initial BOD_5-inhibition above 30% to reach a negligible toxicity at the end of the experiment. Both, BOD_5- and OUR-based data indicate that no significant amounts of toxic intermediates were formed during the photocatalytic process, as a clear relationship was found between toxicity and the concentration of carbaryl.

Finally, the more sensitive assay based on the luminescence of the bacteria *V. fischeri* was also employed. Fig. 2 shows that inhibition was above 90% for the untreated solution and successful detoxification was achieved, reaching close to 30%. Nevertheless, these toxicity tests are usually more sensitive than activated sludge; in particular, 30% of inhibition detected at the end of the treatment

Table 1
Concentration of carbaryl, DOC, and mineralization (expressed as % of DOC removal in photo-Fenton pre-treatment) of the samples tested with *Pseudomonas putida* CECT 324

Sample	1	2	3	4	5	6	7	8	9	10
DOC (mg L^{-1})	76.5	66.5	65.1	61.4	57.2	48.9	44.4	39.9	30.3	18.4
Carbaryl (mg L^{-1})	136	15.3	8.5	2.2	0	0	0	0	0	0
Mineralization (%)	0	13	15	20	25	36	42	48	60	76

is already rather higher than desirable for disposal in the environment, but it could be perfectly alright for combination with a biological system.

A careful study of the chemical analyses and toxicity bioassays indicate that despite the good performance of the photochemical process, a complementary treatment appears convenient: despite differences among sensibility of the methods, all three toxicity assays are coincident to conclude that it is necessary to eliminate the carbaryl so that the toxicity considerably diminishes; however, after carbaryl elimination DOC concentration was still rather high (more than 40% of the initial DOC), being recommendable a complementary biological treatment.

The gross parameter BOD_5 was used as an indicator of the biodegradability of the treated effluent, in order to consider the applicability of a biological treatment to deal with the remaining organic matter. Fig. 3 shows that BOD_5 suffers a sharp increase at the beginning of the photo-oxidative process, followed by a slow decrease that could be probably attributed to the oxidation of the organic matter.

The BOD_5/COD ratio was calculated as this parameter is considered to be a better indicator of biodegradability [3]; it increased to reach values well above 0.5, which is commonly used as a value above which a biological treatment could be considered pertinent. As it was remarked in the discussion about Fig. 1, the maximum AOS was reached at the end of the treatment after approximately 180 min of illumination time; BOD_5/COD ratio reached a maximum approximately at the same moment. Therefore, the importance of AOS for predicting biodegradability is more remarkably after these results as formation of more oxidised intermediates (AOS increase) indirectly demonstrates biodegradability improvement.

3.2. Toxicity and biodegradability test using *P. putida*

To go further on the study of the ability of bacteria present in activated sludge to metabolise the treated effluent, experiments were carried out also with *P. putida*, which is commonly found among the micro-organisms present in activated sludge. Higher initial DOC values were required for those experiments in order to have enough DOC for permitting *P. putida* growth, and for this reason, higher carbaryl concentration and the faster photo-Fenton process was employed as chemical pre-treatment. By this procedure, a rapid degradation of carbaryl could be achieved without an important mineralization due to the high reaction rate achieved by photo-Fenton with aromatic compounds [21]. Data shown in Table 1 indicate that when complete removal of the active ingredient was achieved, DOC was still 57 mg L^{-1}, high enough to perform the required biological assays.

As intermediates formed during the photo-Fenton process could be slightly different to the titania-driven photocatalysis, detoxification of the solution was checked according to the inhibition of the OUR. Before treatment, the corrected inhibition was 65% and, as occurred with the TiO_2 treatment, samples taken after complete elimination of carbaryl were not able to inhibit the respiration of the sludge, and thus, formation of toxic intermediates towards the sludge could be ruled out.

In a first series of experiments, the possible toxicity of carbaryl was studied with *P. putida* in Erlenmeyer flask. In this assay *P. putida* was able to grow on the available degradable carbon source despite the presence of carbaryl in solution. Although some DOC consumption was measured at 96 h of culture, pollutant concentration remained constant at its initial value as determined by HPLC measurement. This result points out that *P. putida* growth is not seriously inhibited by carbaryl, but the bacterium is not able to metabolise this compound.

Samples submitted to different intensities of photocatalytic treatment (Table 1) were assayed in Erlenmeyer flasks as described in Section 2.5. After 96 h of culture, the biodegradation efficiency (E_f) of photo-reaction intermediates was calculated as the ratio between the actual organic carbon uptake and the maximum DOC consumption [12], according to the following equation:

$$E_f = \frac{DOC_i - (DOC_f - DOC_m)}{DOC_i} \times 100 \quad (5)$$

where DOC_i is the initial DOC concentration given by photo-reaction intermediates, DOC_f is the measured DOC concentration at the end of culture and DOC_m is the minimum concentration that cannot be metabolised by the cells, which is the background level reached with the blank medium and accepted as 30 mg L^{-1}.

At the start of phototreatment (sample 1), the efficiency is zero in agreement with toxicity studies showed above, as no interme-

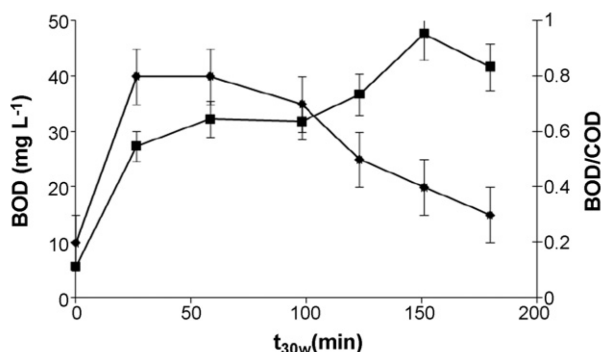

Fig. 3. Plot of the BOD_5 (♦), and BOD_5/COD ratio (■) of an aqueous solution of Sevnol after different periods of treatment.

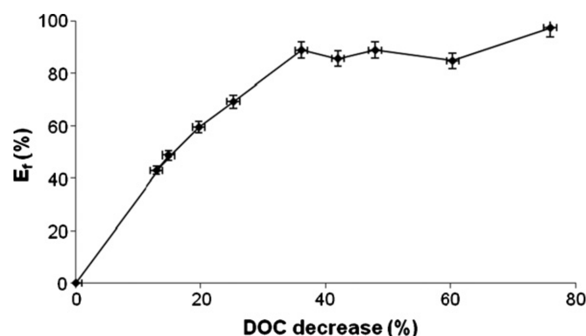

Fig. 4. Efficiency of biodegradation (E_f) on carbaryl photo-treated samples as a function of DOC decrease at 96 h. The standard deviation of E_f was estimated to be 3.6%.

diates from carbaryl were formed and the bacteria growth on the baseline medium. Nonetheless, *P. putida* was able to biodegrade the intermediates generated even when parent carbaryl is still present (samples 2–4, with DOC removal below 30%) although this pesticide was not consumed in any case. This results in an increase in efficiency of the biological treatment with the progress of photo-Fenton reaction in the carbaryl assays. This trend can be observed from 13 to 36% of chemical mineralization (samples 2–6) reaching an efficiency of biological process of 89%. Beyond sample 6, the efficiency is approximately constant with a slight increase to reach 97% in the last sample. A comparison between Figs. 3 and 4 shows that both series of results are in general agreement, showing an increase in the biodegradability of the sample, related with removal of carbaryl and formation of biodegradable intermediates. This proves that *P. putida*-based assays could be an interesting tool to evaluate changes in the biodegradability of an experiment and deserves further research.

4. Conclusions

Determination of the biocompatibility of the organic matter based on *P. putida* is a promising strategy as preliminary results obtained with this bacterium are comparable with those determined using activated sludge. Both methodologies have proven that a solar-driven photo-Fenton process is able to enhance significantly the biocompatibility of an aqueous solution of carbaryl, once this pesticide has been removed, despite the mineralization of the solution was only moderate (36% DOC removal). Based on these results, it could be concluded that assays using *P. putida* might be particularly advantageous, as they are not moderately time consuming and involve a definite living system instead of the complex and variable consortium of micro-organisms as are activated sludge. With these results, more research on the applicability of *P. putida* assay to complex biorecalcitrant effluents is meaningful.

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