



## Supportive use of cyclodextrins as decontamination agents for herbicides

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## Supplement article

# Supportive use of cyclodextrins as decontamination agents for herbicides: the case of fenoxaprop-p-ethyl

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**ABSTRACT:** The inclusion complexes of herbicides in cyclodextrins (CDs) have been considered in an attempt to assess the potentiality of CDs as new formulation additives for both delivery improvement, and reduction of toxicity in terms of environmental impact.

The aim of this work was to evaluate the role of three different types of CDs—namely  $\beta$ -CD and its two derivatives, randomly methylated  $\beta$ -CD and hydroxypropyl  $\beta$ -CD—towards the herbicide fenoxaprop-p-ethyl (FE) properties. The toxicity of both pure FE (either alone or as inclusion complex) and a commercial product has been evaluated by an amperometric biosensor based on a Clark electrode as a respirometry study on *Saccharomyces cerevisiae* strain. Such a technique results more sensitive with respect to FE toxicity methods based on cell growth. The toxic effect of the commercial product was higher than that of the pure chemical entity. Photochemical studies on FE and FE-CD complexes in the presence of different soil colloidal components showed the catalytic effect of some of CDs on photodegradation. The obtained results suggest that CDs could be useful excipients to promote enhancement of solubility, photodegradation inductive effect, and bioremediation in case of FE-like hydrophobic herbicides. © 2012 Curtin University of Technology and John Wiley & Sons, Ltd.

**KEYWORDS:** fenoxaprop-p-ethyl; cyclodextrin; Clark electrode; respirometry study; photodegradation; detoxification; bioremediation

## INTRODUCTION

The current condition of widespread environmental contamination, in Italy as well as generally existing in many other Countries, leads to the need to rationalize the increasingly amounts of pesticides used in agriculture to limit the onset of serious eco-toxicological problems.<sup>[1]</sup> The toxicological requirements prescribed by the European regulatory context do not sufficiently consider the risks associated with co-formulants present in commercial herbicide preparations.<sup>[2]</sup> In fact, they refer only to active ingredients (AI) despite a difference in toxicity between substances either alone or in formulation is well known.<sup>[3,4]</sup> However, the US Environmental Protection Agency regulates the inclusion of certain ingredients in adjuvant formulations, but it does not stringently test and regulate the manufacture and use of adjuvant products (as they do for herbicides and other pesticides).<sup>[5,6]</sup> Actually, if the so-called inert compounds such as surfactants, co-solvents, stabilizers, preservatives, and buffers, improve the AI properties (such as solubility, penetration, efficacy, chemical and thermal stability, biodegradability, smell,

volatility, soil mobility, persistence, and poor wettability), on the other hand, they may amplify its adverse effects.<sup>[7,8]</sup> It is therefore necessary to evaluate the toxicity of the formulations as a whole rather than focusing only on AI and to propose new functional ingredients capable of improving the AI properties without increasing its toxicity profile, but if anything, minimizing the toxicity.

For this reason, the development of new formulations for herbicides represents therefore an important resource to rely on to fulfill the AI effectiveness, resulting in both dose limitation and significant reduction of the environmental dispersion of the used product.

Cyclodextrins (CDs), cyclic oligosaccharides able to host hydrophobic molecules in the toroidal interior part, by complexing pesticides can result in products with superior performance as carrying agents of pesticides.<sup>[9]</sup>

In the field of environmental chemistry, CDs promote the degradations of organic pollutants through the enhancement of their solubility and, therefore, their desorption from soils.<sup>[10,11]</sup> In detail, inclusion complex results in advantageous modifications of the properties of the complexed substances, such as enhancement of wettability, rate of dissolution, solubility, and long lasting effect.<sup>[12]</sup> Furthermore, the effect of CDs other than on increasing water solubility of some herbicides can be observed on their photodegradation, possibly in the presence of soil components.<sup>[13]</sup> The study of the

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photolysis of pesticides is important in environmental chemistry because of its influence on their fate in terms of persistence in natural sunlight.<sup>[12]</sup>

In a previous study,<sup>[14]</sup> the toxicity of various pesticides, including fenoxaprop-p-ethyl (FE, Fig. 1), an aryloxyphenoxypropionate post-emergence herbicide inhibiting fatty acid synthesis in grasses,<sup>[15,16]</sup> as both pure AI and commercial product, has been evaluated using a cell growth method. The obtained results showed that pure FE affected at a lower extent cell growth and metabolism with respect to its commercial preparations containing various additives, capable of imparting significant major negative influence on yeast biological parameters.<sup>[14]</sup>

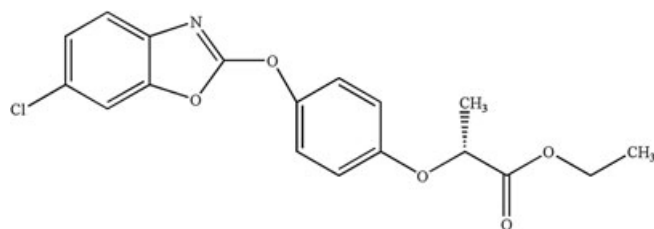
On the other hand, Zhang *et al.* almost simultaneously characterized the interactions of CDs with FE, highlighting how these are capable of forming complexes with FE, thus not only enhancing the solubility of FE for more efficient delivery, but also preventing its hydrolysis to facilitate its penetration into plant cells.<sup>[17]</sup> Consequently, it is also possible to provide for the use of CDs, especially random methylated  $\beta$ -CD and hydroxypropyl- $\beta$ -CD, in FE formulations as common enhancing additives in the bioremediation of soils.<sup>[18]</sup>

In the present paper, the complexation of herbicide FE, with natural and synthetic CDs was therefore investigated, as well as the toxicity of FE either alone, or in combination with CDs or even in commercial formulation. The possible influence of inclusion complexes on the photodegradation, still in the presence of soil constituents, has been also studied. For such purposes, goethite and bentonite have been selected because of their pro-oxidant and adsorbent properties, respectively.<sup>[19–22]</sup>

## MATERIALS AND METHODS

### Reagents and chemicals

*Saccharomyces cerevisiae* (Zymasil,  $3.0 \cdot 10^{10}$  cells/g) was purchased from Pascal Biotech (Italy); D (+)-Glucose Monohydrate, Fenoxaprop-p-ethyl (FE, Pestanal<sup>®</sup>), Goethite, and Bentonite from Sigma-Aldrich (Germany), whereas proper energy (PE) was from commercial sources (Aventis CropScience, Milan, Italy);  $\beta$ -CD



**Figure 1.** Structural formula of fenoxaprop-p-ethyl.

and hydroxypropyl- $\beta$ -CD ( $\beta$ CD and HP $\beta$ CD, Kleptose and Kleptose HPB, respectively) were the generous gift from Roquette (France) and randomly methylated  $\beta$ -CD (RAMEB, Cavasol) from Wacker Chemie AG (Germany). Freshly distilled water was used throughout the experiments.

### Phase-solubility studies

Phase-solubility studies were performed according to the method reported by Higuchi and Connors.<sup>[23]</sup> FE, in constant amounts exceeding its solubility, was transferred to vials containing 10 mL of different CDs in pH 6.8 phosphate buffer 0.15 M ( $\beta$ CD ranging from 0 to 16 mM; HP $\beta$ CD and RAMEB ranging from 0 to 100 mM). The contents were stirred (400 rpm) on electromagnetic stirrer (VELP Scientifica, Italy) at  $37 \pm 0.5^\circ\text{C}$  for 7 days. After reaching equilibrium, samples were filtered through a 0.45- $\mu\text{m}$  nylon membrane filter (Millipore, USA). The filtered samples suitably diluted were assayed for FE by measuring absorbance at the wavelength of 239 nm (Lambda 25 UV/VIS, Perkin Elmer, Milan, Italy). The analytical method has been validated for the quantitative determination of the analyte at the experimental conditions adopted by coefficient of determination of the relative calibration curves ( $r^2 \geq 0.9998$ ). Solubility studies were performed in triplicate. The stability constant of the inclusion complex ( $K_c$ ) were calculated from the phase solubility diagrams using Equation 1:

$$K_c = \frac{k}{S_0(1 - k)} \quad (1)$$

where  $S_0$  (intercept value) is the intrinsic solubility of FE in the absence of CD, and  $k$  is the slope of the straight line.

All the measurements were performed five times, unless otherwise stated.

### Toxicity studies

The measurement of oxygen consumption in solution due to biochemical-physiological process of cellular respiration<sup>[24]</sup> has been obtained by deeping a Clark electrode, connected to a potentiostat Amel 2059 (Amel srl, Milan, Italy) and in thermostatted ( $37 \pm 0.5^\circ\text{C}$ ) *Saccharomyces cerevisiae* suspensions (0.15 mg/mL, pH 6.8 phosphate buffer 0.15 M, glucose 9.9 mg/mL), under stirring conditions,<sup>[25]</sup> in the absence or in presence of either pure FE or PE as toxic agents ( $1.5 \cdot 10^{-4}$ – $7.2 \cdot 10^{-2}$  mM). In the case of pure FE, ethanol has been added as co-solvent (5%v/v final). It is assumed that a decrease in respiratory activity of cells is related to the damage suffered by the exposed cells and then the index of respiratory inhibition ( $I_{RI}$ ) was calculated using Equation 2:

$$I_{RI} = 1 - \frac{r}{r_0} \quad (2)$$

where  $r$  is the respiratory activity with herbicide and  $r_0$  is the respiratory activity without herbicide.

All the measurements were performed five times, unless otherwise stated.

### Photodegradation study

Buffered FE solutions (pH 6.8, 2.77 mM) were exposed at a distance of 10 cm under aerobic conditions and constant stirring to a Photochemical Multirays Apparatus (ten lamps,  $\lambda \geq 310$  nm, 15 W, Helios Italquartz, Milan, Italy) at room temperature ( $25 \pm 0.5^\circ\text{C}$ ) at the following different conditions: (i) as is; (ii) in the presence of CDs in pH 6.8 phosphate buffer 0.15 M ( $\beta\text{CD}$  16 mM; HP $\beta\text{CD}$  or RAMEB, 10 and 100 mM); iii) in pH 6.8 buffered suspension of colloidal soil components (0.08 mg/L of either goethite or bentonite), in the absence and in presence of RAMEB 100 mM. Samples withdrawn at fixed time intervals (0–180 min) were filtered and spectroscopically analyzed as previously reported, to monitor the CD-induced effects on the photodegradation process. The results are expressed as the ratio among spectroscopic variations. Control experiments were carried out in the darkness.

All the measurements were performed five times, unless otherwise stated.

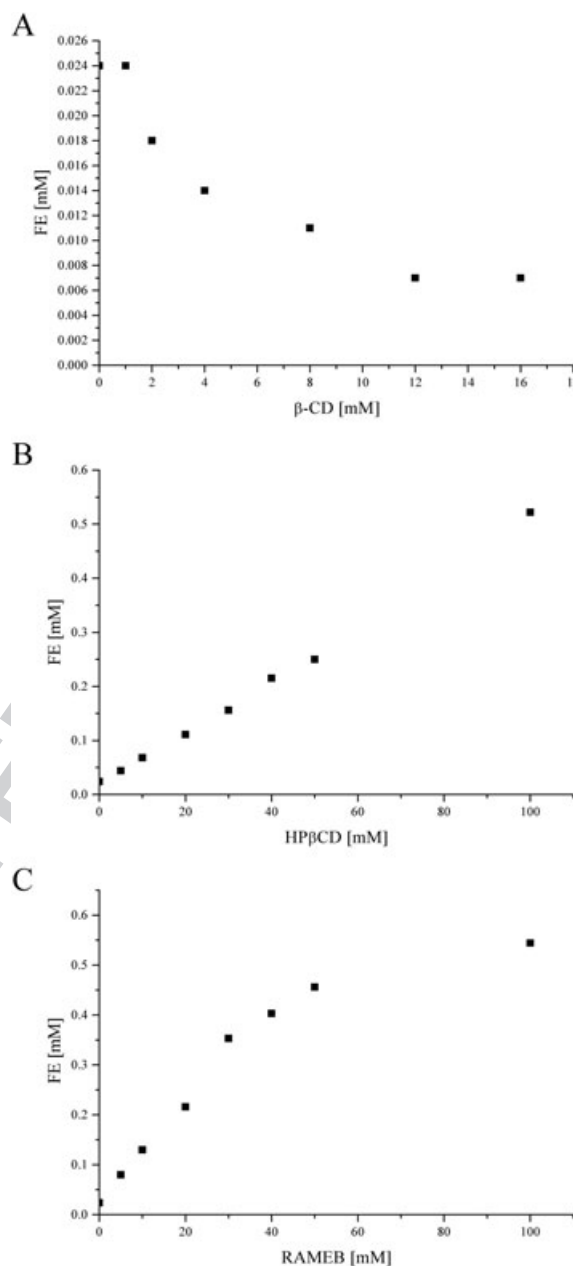
### Statistical analysis

Results are expressed as the mean values ( $\text{CV}\% < 2$ ), unless the bar errors have been reported ( $\pm\text{SD}$ ). One-way ANOVA performing the Bonferroni post-test (InStat software, version 3.0 GraphPAD Software Inc., San Diego, CA) was used for the statistical analysis of the results. Significance was defined as a  $p$ -value less than 0.05.

## RESULTS AND DISCUSSION

**F2** The solubility phase diagram of FE is reported in Fig. 2. Panel A shows an unusual pattern because of the presence of the natural  $\beta\text{CD}$ . It follows the  $B_1$  model described by Higuchi and Connors, characteristic of insoluble complexes, according to which there is an immediate and progressive decrease in the solubility of the molecule complexed with increasing concentrations of CD.<sup>[23]</sup>

On the other hand, the presence of HP $\beta\text{CD}$  results in a linear increase of FE solubility (Fig. 2, panel B), with  $K_{1:1(\text{HP}\beta\text{CD})} = 204 \text{ M}^{-1}$  as the calculated complex stability. Even in the case of RAMEB (Fig. 2, panel C), it has been shown a proportional trend of FE solubility with respect to RAMEB. Such an increase was linear



**Figure 2.** Solubility studies of FE in the presence of  $\beta\text{CD}$  (panel A), HP $\beta\text{CD}$  (panel B), and RAMEB (panel C). The coefficient of variation was always less than 5%.

until the concentration of 30 mM, whereas at concentrations above the trend conforms to the type  $A_N$ .<sup>[23]</sup> It represents a typical case of self-associate complexes and is potentially caused by alteration of the solvent properties as a result of the use of high concentrations of inclusion complexation agent. As for the linear segment, it has been possible to evaluate  $K_{1:1(\text{RAMEB})} = 440 \text{ M}^{-1}$ , showing a greater complexing power of RAMEB than HP $\beta\text{CD}$ , with a greater and more rapid significant increase in solubility. Otherwise, at the maximum CDs concentration, the relative solubility increase is similar at the experimental conditions adopted.

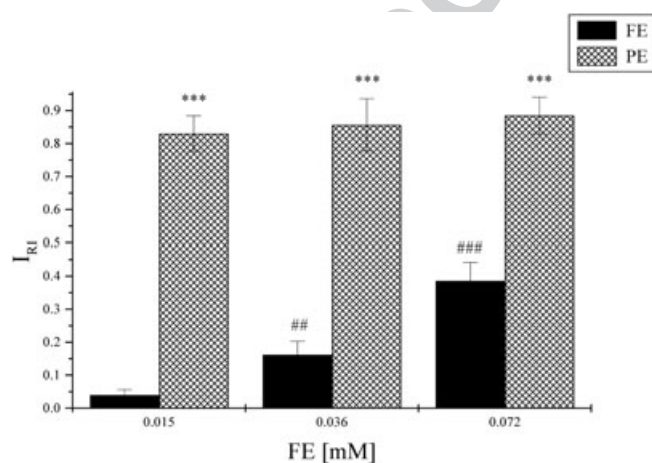
Compared with what has been previously published operating at lower concentrations of CDs ( $\leq 10$  mM) and at lower temperatures ( $25$  °C),<sup>[17]</sup> it is clear that the solubility of FE has shown higher values in our conditions, as well as K values for each CD were lower, as expected for an exothermic process.<sup>[26,27]</sup>

The toxicity of FE either alone or as PE formulation is shown in Fig. 3 in terms of  $I_{RI}$ .

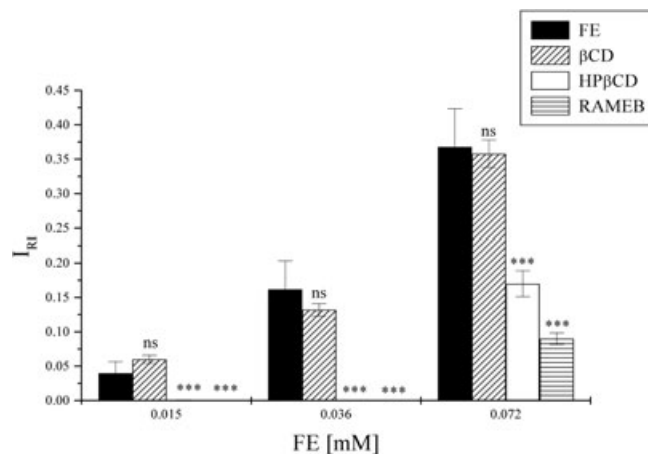
Although for FE concentrations  $\leq 1.5 \cdot 10^{-3}$  mM, both as pure substance as well as in formulation, the toxicity appears to be negligible (data not shown), starting from  $1.5 \cdot 10^{-2}$  mM, FE shows a lower toxicity than PE, with maximum values of  $I_{RI}$  of about 0.4 at  $7.2 \cdot 10^{-2}$  mM for FE with respect to at least 0.8 at  $1.5 \cdot 10^{-2}$  mM for PE.

This result shows that the toxic effect of the formulated product is markedly higher than the pure herbicide because of the synergy of the ingredients. The comparison with previously published data by carrying out toxicity tests with inhibition of cell growth showed a higher sensitivity of the present amperometric method. In particular, the pure compound provided a concentration-dependent toxicity expressed in terms of  $I_{RI}$  with values ranging from 0.04 to 0.39 vs the absence of toxicity (no inhibition) previously reported.<sup>[14]</sup> Also in the presence of excipients of the commercial PE, differences as obtained by the two methods have been confirmed, obtaining in the case of  $1.5 \cdot 10^{-2}$  mM an  $I_{RI}$  of 0.81 vs a toxicity index of 0.36 as extrapolated by the speed variation of cultured cells growth. At the maximum AI concentration studied ( $7.2 \cdot 10^{-2}$  mM), the differences in toxicity are less evident (0.88 and 0.81, respectively).

Moreover, the toxicity of FE in terms of  $I_{RI}$  has been tested in pH 6.8 buffered solution, in the presence of either  $\beta$ CD (16 mM) or HP $\beta$ CD (100 mM), or RAMEB (100 mM). The results are shown in Fig. 4.



**Figure 3.** Index of respiratory inhibition ( $I_{RI}$ ) as evaluated for both pure fenoxaprop-p-ethyl (FE) and in commercial formulation proper energy (PE), at different concentrations. Statistical analysis: two symbols,  $p < 0.01$ ; three symbols,  $p < 0.001$ ; star symbol, FE vs PE; sharp symbol, effect of concentration.

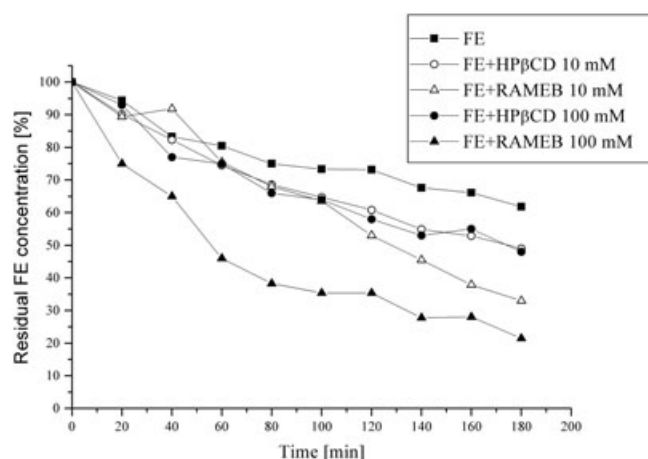


**Figure 4.** Influence of cyclodextrins (CDs) in relation to fenoxaprop-p-ethyl (FE) toxicity by index of respiratory inhibition ( $I_{RI}$ ). Statistical analysis: ns, not significant; three stars,  $p < 0.001$ .

It is evident that the presence of CDs in the solution reduces the exposure of *S. cerevisiae* to FE. In particular, the protection with respect to the toxic effect as evaluated by respirometric assay decreases in the order: RAMEB > HP $\beta$ CD >>  $\beta$ CD.

Regarding the studies of photodegradation, the FE solutions when light-irradiated undergo photodegradation, as shown in Fig. 5 where the % of residual FE, in the presence and absence of the CDs previously observed as shield compounds in terms of toxicity, namely RAMEB and HP $\beta$ CD, are plotted as a function of exposure time.

The degradation profiles of FE alone in aqueous solution buffered to pH 6.8 show a discrete reduction of the concentration of the herbicide with a residual percentage of FE, after 180 min of irradiation, of 61.8%. The presence of CD increases the rate of photodegradation. In



**Figure 5.** Degradation profile of fenoxaprop-p-ethyl (FE) at pH 6.8 buffered solution, in the absence and in presence of cyclodextrins (CDs) at various concentrations. The coefficient of variation was always less than 5%.

detail, in the presence of HP $\beta$ CD, the degradation profile is similar at the two concentrations under investigation, and it is practically over impossible to that of RAMEB at the lower concentration. On the contrary, RAMEB at the highest concentration shows a remarkable and fast decrease until a degradation of about 80% after 180 min of treatment. From a mathematical point of view, the degradation profiles in the different experimental conditions follow a first-order kinetic, and both the degradation constants,  $k_{\text{deg}}$  and the half-lives ( $t_{1/2}$ , min), have been calculated. They are reported in Table 1.

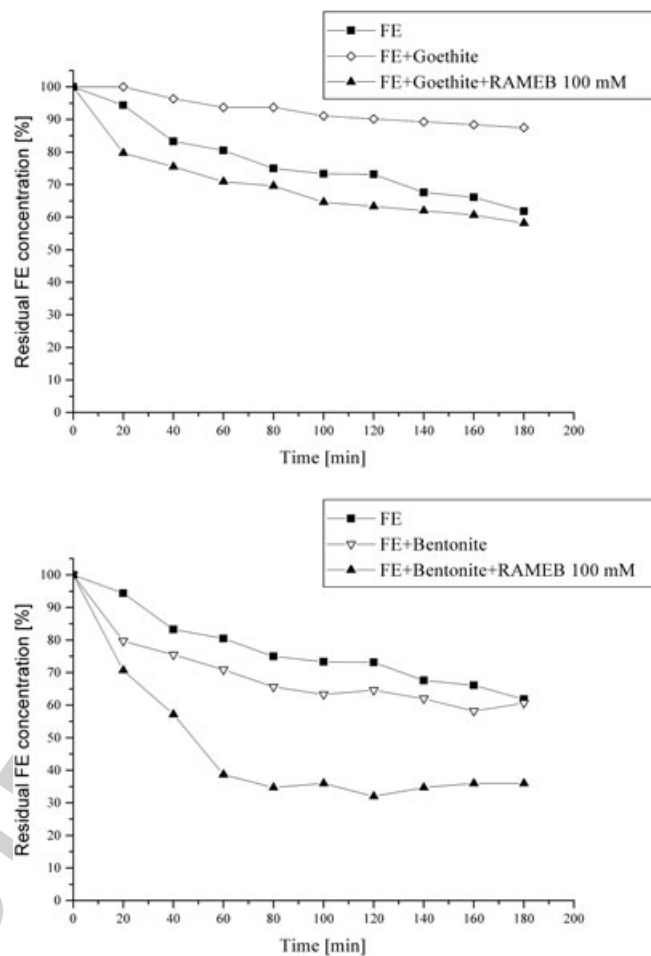
From these results, it is possible to say that RAMEB and HP $\beta$ CD act as catalysts in the photodegradation of FE, ensuring a higher rate of degradation in the light and thus less of the toxic compound remaining in the soil. It is also evident that there is a clear correlation between photodegradation and characteristics of the complex with CDs: the higher the value of  $K_{1:1}$ , the greater the stability of the binary system, with a greater photocatalytic effect.<sup>[28]</sup>

The presence of colloidal components of the soil can vary the photodegradation of herbicide compounds, reducing eco-toxicological characteristics.<sup>[13,29]</sup> To mimic the environmental conditions, the photodegradation studies were also conducted in buffered solution in the presence of different soil colloidal components. In particular, two components of the soil were chosen: goethite and bentonite, both tested at a concentration of 0.08 mg/mL. In particular, goethite suspensions may act to form singlet oxygen in aqueous environment systems leading to effective oxidation of pollution matters,<sup>[22]</sup> whereas bentonite differently adsorb chemical entities.<sup>[19,20]</sup> Because of its favorable complex characteristics, 100-mM RAMEB was also chosen to test the influence of CD complexation on the degradation kinetics in the presence of these colloidal constituents. The remaining% of FE, in the presence and absence of colloidal components in the suspension buffer and in the presence and absence of RAMEB, is plotted as a function of exposure time in Fig. 6, whereas the corresponding degradation constants and  $t_{1/2}$  are reported in Table 2.

The presence of goethite causes a significant reduction in herbicide photodegradation with a residual 87.5% after 180 min of irradiation, and a  $t_{1/2} = 826.1$  min compared with 243.2 min of buffered aqueous solution. Such a result suggests a photoprotection by this colloidal component

**Table 1. Degradation constants,  $k_{\text{deg}}$  and half-life,  $t_{1/2}$  of FE in the presence of HP $\beta$ CD or RAMEB at the different experimental conditions.**

Samples	$k_{\text{deg}} \cdot 10^{-3} \text{ (min}^{-1}\text{)}$	$t_{1/2} \text{ (min)}$
FE	$2.85 \pm 0.12$	243.2
FE + HP $\beta$ CD 10 mM	$4.24 \pm 0.10$	163.5
FE + RAMEB 10 mM	$5.72 \pm 0.19$	121.2
FE + HP $\beta$ CD 100 mM	$4.29 \pm 0.16$	161.6
FE + RAMEB 100 mM	$9.17 \pm 0.43$	75.6



**Figure 6.** Degradation profile of fenoxaprop-p-ethyl (FE) at pH 6.8 buffered solution, in the presence of either goethite (top) or bentonite (bottom), with or without RAMEB. The coefficient of variation was always less than 5%.

probably due to adsorption of FE on the surface of the goethite with a consequent shielding effect.<sup>[13]</sup> The addition of 100-mM RAMEB to the aforementioned colloidal suspension led to a much more pronounced FE photodegradative profile (Fig. 6, top), even if such an increase did not reach the same values of  $k_{\text{deg}}$  and  $t_{1/2}$  obtained in the absence of goethite ( $3.63 \text{ min}^{-1}$  vs  $9.17 \text{ min}^{-1}$  and  $190.9 \text{ min}$  vs  $75.6 \text{ min}$ , respectively). This indicates the possible occurrence of competitive phenomena between both the photoprotective and the photocatalytic effects by goethite and RAMEB. A deeper study of these phenomena is under investigation, and it has been considered beyond the aim of the present paper.<sup>[30,31]</sup>

On the other hand, the kinetic of FE photodegradation in the presence of bentonite has shown a trend almost similar to that achieved in only phosphate buffer at pH 6.8. Such a result suggests a lack of photoprotective effect of bentonite. The addition of 100-mM RAMEB to FE suspension facilitates and noticeably promotes the photocatalytic degradation of FE, obtaining a residual percentage 36% (Fig. 6, bottom). Moreover, for exposure

**Table 2. Degradation constants,  $k_{\text{deg}}$  and half-life,  $t_{1/2}$  of FE in the presence of colloidal components of the soil, with or without RAMEB.**

Samples	$k_{\text{deg}} \cdot 10^{-3} \text{ (min}^{-1}\text{)}$	$t_{1/2} \text{ (min)}$
FE	$2.85 \pm 0.12$	243.2
FE + Goethite	$0.839 \pm 0.03$	826.1
FE + Goethite + RAMEB 100 mM	$3.63 \pm 0.31$	190.9
FE + Bentonite	$3.62 \pm 0.32$	191.5
FE + Bentonite + RAMEB 100 mM	$K_{\text{lim}} = 34.03 \pm 2.16$ $C_{\text{lim}} = 33.15\% \pm 1.34$	$t_{1/2(\text{lim})} = 20.4$

times <60 min, the FE degradation shows a velocity much higher than the system without CD, as if it were able to act in synergy and increase the mildly photocatalytic ability exhibited by bentonite, because of a concentration-dependent modification by RAMEB of its surface, pore, and aggregate properties.<sup>[32]</sup> Such a dual effect can be explained by the combination of the shielding properties of the bentonite with its ability to produce radical species on the surface adsorption.

## CONCLUSION

In conclusion, the complex formation of herbicides with suitable CDs can be a useful tool for bioremediation of soils. In particular, the adopted technique has proven a very sensitive method in terms of toxicity evaluation at different potential contamination conditions, and it advances the knowledge of these applicative fields. As for the toxicity studies, the adaptation of the FE respirometry study on the effect of colloidal components and CDs is an area that still requires further investigation and understanding, and it will be evaluated in future studies. Analogously, the toxicity studies of both commercial products and degradation products in the presence of CDs with and without CDs are potential and attractive developments of this work.

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**Article: apj\_1654**

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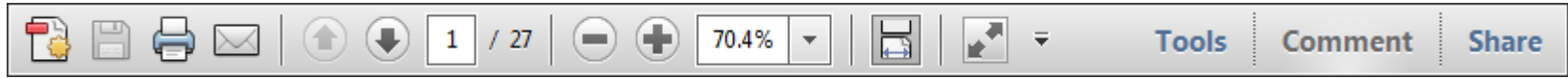


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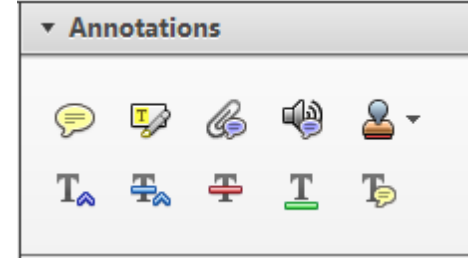
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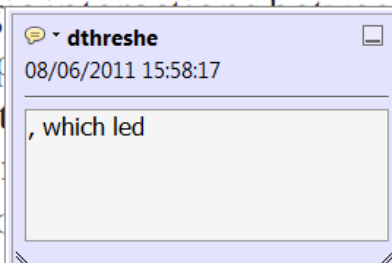


Strikes a line through text and opens up a text box where replacement text can be entered.

**How to use it**

- Highlight a word or sentence.
- Click on the [Replace \(Ins\)](#) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

standard framework for the analysis of microeconomics. Nevertheless, it also led to the emergence of a new paradigm of strategic behaviour. The number of competitors in the industry is that the structure of the industry is a key component of the main components of the industry. At the micro level, are exogenous variables important works on entry by Shiraz (M henceforth) we open the 'black b



**2. Strikethrough (Del) Tool – for deleting text.**



Strikes a red line through text that is to be deleted.

**How to use it**

- Highlight a word or sentence.
- Click on the [Strikethrough \(Del\)](#) icon in the Annotations section.

there is no room for extra profits and the number of competitors are zero and the number of competitors (net) values are not determined by the number of firms. Blanchard and ~~Kiyotaki~~ (1987), perfect competition in general equilibrium. The effects of aggregate demand and supply in the classical framework assuming monopoly. The number of firms is an exogenous number of firms

**3. Add note to text Tool – for highlighting a section to be changed to bold or italic.**



Highlights text in yellow and opens up a text box where comments can be entered.

**How to use it**

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

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**4. Add sticky note Tool – for making notes at specific points in the text.**

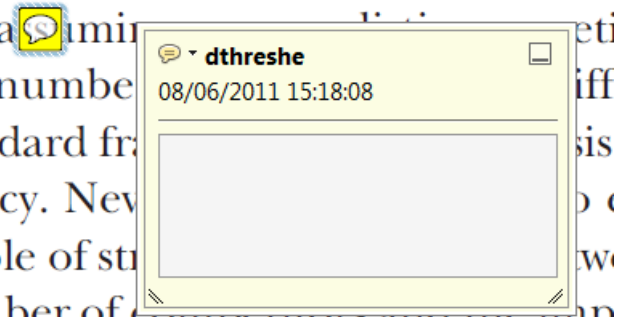


Marks a point in the proof where a comment needs to be highlighted.

**How to use it**

- Click on the [Add sticky note](#) icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

and supply shocks. Most of the... a... number... dard fr... cy. Nev... ole of st... ber of competitors and the imp... is that the structure of the secto



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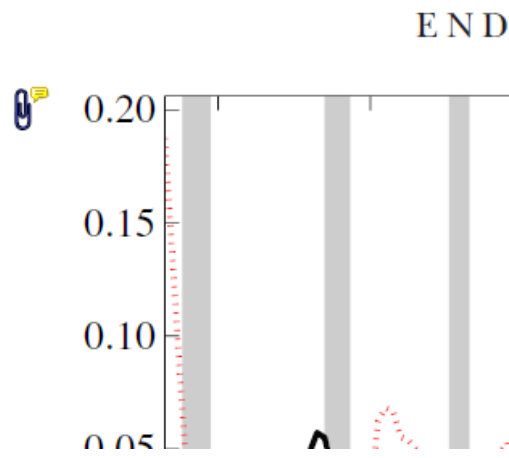
**5. Attach File Tool – for inserting large amounts of text or replacement figures.**



Inserts an icon linking to the attached file in the appropriate place in the text.

**How to use it**

- Click on the [Attach File](#) icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



**6. Add stamp Tool – for approving a proof if no corrections are required.**



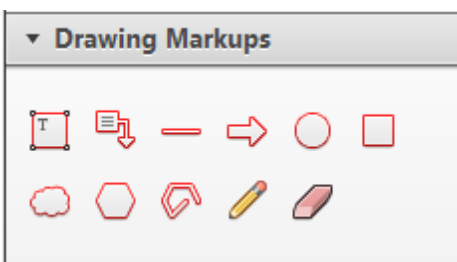
Inserts a selected stamp onto an appropriate place in the proof.

**How to use it**

- Click on the [Add stamp](#) icon in the Annotations section.
- Select the stamp you want to use. (The [Approved](#) stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

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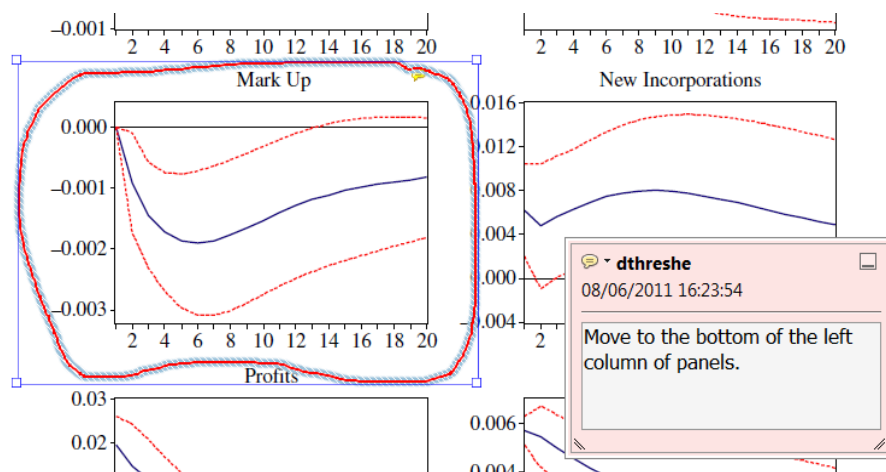


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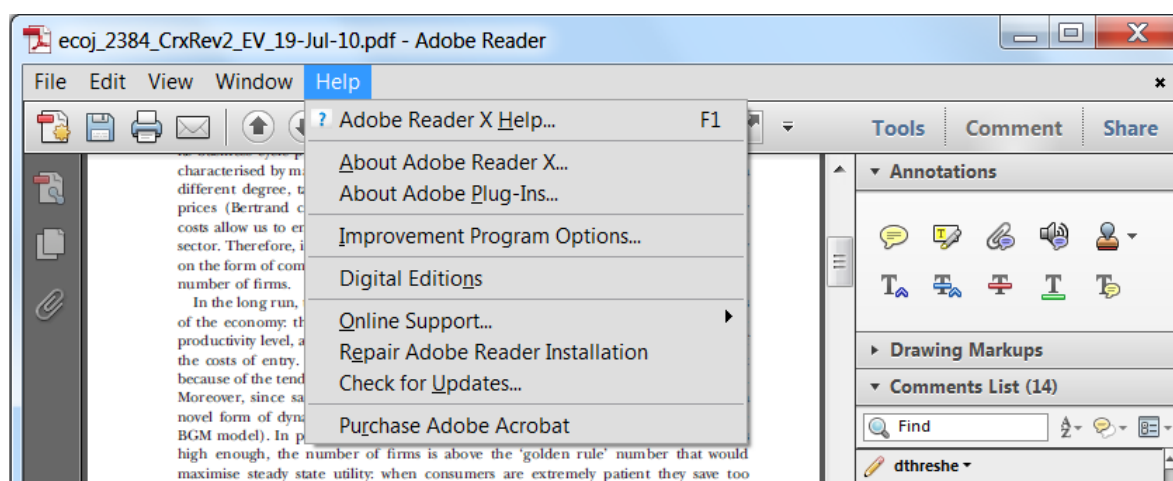
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

**How to use it**

- Click on one of the shapes in the [Drawing Markups](#) section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



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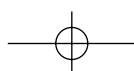
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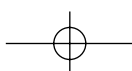
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