

## TiO<sub>2</sub> PHOTOCATALYTIC DEGRADATION OF CAFFEINE AND ECOTOXICOLOGICAL ASSESSMENT OF OXIDATION BY-PRODUCTS

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### ABSTRACT

The photocatalytic degradation of caffeine has been investigated in aqueous suspensions of titanium dioxide (TiO<sub>2</sub>) by monitoring process efficiency at varying TiO<sub>2</sub> (29.3 – 170.7 mg l<sup>-1</sup>) and initial drug concentrations (0.76 - 9.24 mg l<sup>-1</sup>). The use of the response surface methodology (RSM) allowed to fit the optimal regions of the parameters leading to the degradation of the pollutant. Also, a single polynomial expression modelling the reaction was obtained. In parallel a set of bioassays (*Daphnia magna*, *Pseudokirchneriella subcapitata* and *Lactuca sativa*) was performed to evaluate the process in terms of detoxification of caffeine oxidation by-products under chronic and acute tests. Results clearly demonstrate that caffeine is quickly degraded, but not mineralized as quickly, and that persistent toxic organic intermediates resist further oxidation.

**Keywords:** caffeine, *Daphnia magna*, *Lactuca sativa*, photocatalysis, *Pseudokirchneriella subcapitata*

### 1. Introduction

The widespread occurrence of pharmaceuticals in surface waters attests their lack of efficient removal during wastewater treatment and/or their relative persistence in receiving waters. Depending on local industry and land-use practices, pharmaceutically active compounds can be introduced into aquatic systems by industrial waste, animal husbandry practices, or municipal and domestic wastewater (Lin *et al.*, 2008; Sauv  *et al.*, 2012).

By far caffeine is ranked as the number one drug worldwide, with a massive production of hundreds of tons. Usually employed as a stimulant, caffeine is commonly found in coffee, tea, chocolate, cocoa, and soft drinks (Potera, 2012). It is also a component of hundreds of prescription drugs, ranging from analgesics to cold medicines. The half-life of caffeine in humans is on the order of 3.5 to 5 hours. Although the human body metabolizes this stimulant efficiently, between 0.5 and 10.0% is excreted, mostly in the urine (Ferreira, 2005). As consequence the predominant source of caffeine introduced into the wastewater treatment system is considered the disposal of the unconsumed coffee and caffeinated soft drinks. The presence of caffeine has also been reported in samples collected upstream of sewage treatment plants (STP) discharges, as well as persistent concentrations downstream, a distribution

pattern different from other organic pollutants and probably linked to the existence of additional sources such as unauthorized discharges (Rosal *et al.*, 2009). A recent study indicates that measuring caffeine in municipal water systems provides a good estimate of fecal contamination caused solely by human, because agricultural and industrial sources of fecal coliforms generally do not release caffeine into the environment, however researchers have also demonstrated its potential for use as a deterrent to a variety of agricultural pests (Hollingsworth *et al.*, 2005; Moore *et al.*, 2008). Furthermore, the overall consumption of caffeine means that where there is human sewage, there almost certainly will be caffeine. Sauv  *et al.*, 2012 showed as an arbitrary threshold of 400 ng caffeine l<sup>-1</sup> allows to identify samples with an elevated fecal contamination, as defined by more than 200 colony-forming units per 100 ml (cfu 100 ml<sup>-1</sup>) of fecal coliforms.

Due to its high solubility (21.7 g l<sup>-1</sup>), negligible volatility and continuous inputs into the sewage system through many anthropogenic sources, caffeine is likely to persist in water. Concentrations of respectively 294 and 1.3 µg l<sup>-1</sup> have been detected in influent and effluent of STP (Trov  *et al.*, 2013), while concentrations of 1.1 – 106 and 0.22 µg l<sup>-1</sup> were found respectively in raw and drinking water (Sodr  *et al.*, 2010).

Although caffeine is not yet a threat on a large scale, further research is needed on its occurrence in natural waters and its chronic toxicity to aquatic organisms. It has been reported that a single dosage of 5 to 10 g of caffeine in humans can be lethal, and caffeine intoxication (or caffeinism) can be observed in single dose of 250 mg (Price, 1990). Symptoms of caffeinism include anxiety, restlessness, sleep disruption, and abnormally rapid heart rate (Heishman *et al.*, 1992). With the potency of caffeine toxicity firmly established, it becomes necessary to evaluate the risk posed by its widespread environmental occurrence.

The use of advanced oxidation processes (AOPs) for the removal of drugs represents a promising alternative to the conventional treatments. Previous works have shown the efficiency of these processes in the abiotic degradation of caffeine, such as sunlight irradiation (Andreozzi *et al.*, 2003), UV/H<sub>2</sub>O<sub>2</sub> (Shu *et al.*, 2013), photo-Fenton reactions (Trov  *et al.*, 2013) and TiO<sub>2</sub>/UV (Marques *et al.*, 2013), electrochemical oxidation (Indermuhle *et al.*, 2013), ozonation (Rosal *et al.*, 2009). Among them, the photocatalysis process has gained growing attention due to the possibility of using solar energy as radiation source, significantly reducing the demand for energy and avoid pH adjustments as commonly required by Fenton and photo-Fenton processes. It is known that upon UV irradiation, TiO<sub>2</sub> particles generate electron–hole pairs (e<sup>-</sup><sub>CB</sub>/h<sup>+</sup><sub>VB</sub>) in the bulk semiconductor which can migrate to the surface to form oxidising species (HOO•, O<sub>2</sub>•<sup>-</sup> and then •OH radicals) via reacting with preadsorbed O<sub>2</sub>. These radical species possess a potential to oxidise organic molecules at the TiO<sub>2</sub> surface. Although the main pathway of photomineralisation may easily be summarised by the reaction below, the detailed mechanisms of the photocatalytic oxidation processes at the TiO<sub>2</sub> surface keep to be elusive, particularly about the initial steps involved in the reactions of HOO•, O<sub>2</sub>•<sup>-</sup> and •OH radicals with the organic compounds (Bekbolet *et al.*, 2002):



The present study focused on the effects of parameters such as TiO<sub>2</sub> and contaminant concentrations on degradation rate of caffeine by photocatalysis. Preliminary information on kinetic were obtained before to set experiments with an experimental factorial design according to the response surface methodology (RSM).

It is well known that some by-products (intermediates) can be formed as result of partial oxidation and the effluent may become more toxic than the untreated solutions or the parent compounds, respectively. Therefore the overall efficiency of the treatment process for this class of chemical pollutants strictly depends on the toxicity and oestrogenic potency assays of treated effluents (Lofrano *et al.*, 2014).

In order to evaluate how biological assays and chemicals analysis agree in the TiO<sub>2</sub> photocatalytic oxidation of caffeine, a set of ecotoxicological assays was performed for each sample, using three different bioindicators: the seeds of the plant *Lactuca sativa*, the microalga *Pseudokirchneriella subcapitata* and the crustacean *Daphnia magna*.

This research not only provides information for predicting the degradation process of caffeine under the related constraints conditions but also highlights the need of biological assays to properly deals with drugs pollution management.

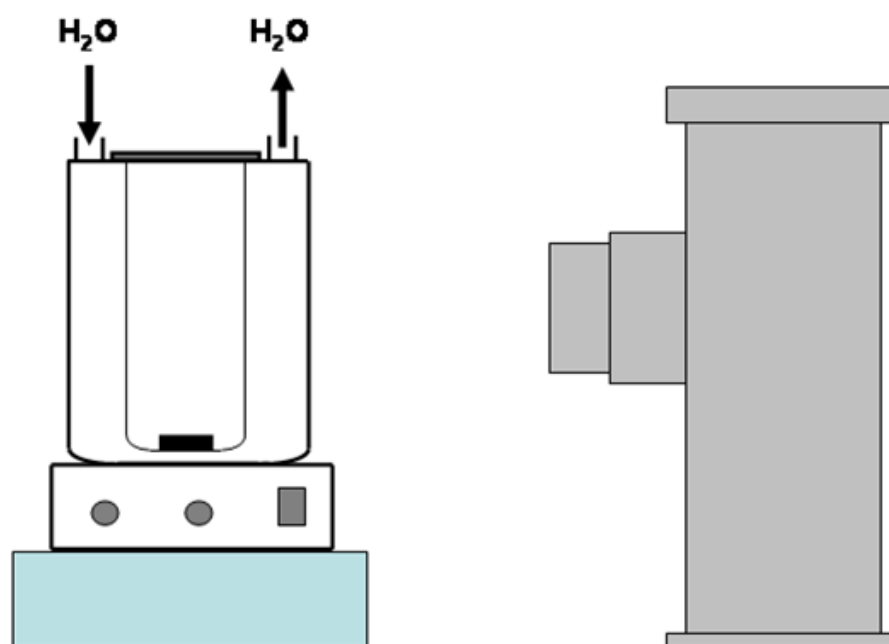
## 2. Materials And Methods

### 2.1. Chemicals

Caffeine, HPLC grade water and methanol were supplied from Sigma Aldrich and used as received without further purification. TiO<sub>2</sub> P25 was kindly furnished by Evonik. Distilled water was used to prepare the caffeine solutions in all experiments.

### 2.2. Photocatalysis experiments

Photocatalysis experiments were performed in a photoreactor equipped with a 400 mL stirred cylindrical Pyrex vessel filled with 200 ml of sample and a xenon arc lamp (450 W, LotOriell Group, Italy) with special glass filters, restricting the transmission of wavelengths above 300 nm (Figure 1).



**Figure 1.** Photocatalytic process experimental setup

The light intensity as determined by the potassium ferrioxalate actinometry was found to be  $4.5 \times 10^{-7}$  Einstein  $s^{-1}$  (Hatchard, 1956).

In photocatalysis experiments, different concentrations of TiO<sub>2</sub> (as shown in Table 1) were added to the solution at natural pH (6.3 - 6.5). The suspension was mixed for 3 min before starting the experiment.

During the kinetic experiments samples of 1.0 ml were withdrawn from the reactor at specific time intervals. Before analysis all samples were filtered through polypropylene syringe filters (0.45  $\mu$ m) to remove TiO<sub>2</sub>.

## 2.3. Analytical procedures

### 2.3.1. UV VIS analysis

Absorption spectra measurements of caffeine solutions were performed on an UV spectrophotometer (Varian Cary50) using 10.0 mm path length quartz cells (Hellma Analytics). The wavelengths at 274.9 nm (UV<sub>274.9</sub>) corresponding to the maximum light absorption of caffeine and at 254 nm wavelength (UV<sub>254</sub>) to characterize the formation of intermediate oxidation compounds having double bonds were recorded.

### 2.3.2. Liquid Chromatography

Changes in the concentration of caffeine were monitored by HPLC-UV (Finnigan Surveyer) equipped with a reversed phase C18 analytical column (Vydac, 5  $\mu$ m, 150 mm  $\times$  3.0 mm). The compounds were separated using as mobile phase a mixture of methanol/ultrapure water (35%/65%) at flow rate of 1 ml min<sup>-1</sup>. The injection volume was 10  $\mu$ L and the wavelength set for the quantification was 274.9 nm. The limit of quantification (LOQ) of this method was 0.5  $\mu$ g ml<sup>-1</sup>.

### 2.3.3. Multivariate experimental design

An experimental factorial design of the reaction system was performed to find the optimal conditions of caffeine degradation during the photocatalytic treatment. Multivariate design was performed according to the methodology of response surface (RSM). The use of factorial design allows finding with minimum number of experiments the optimal experimental conditions for the degradation of a pollutant. The initial caffeine concentration range was considered between 0.76 and 9.25 mg l<sup>-1</sup> while TiO<sub>2</sub> concentration range varied from 29.3 to 170.7 mg l<sup>-1</sup>. The residual caffeine concentration obtained after 5 min of illumination during the photocatalytic treatment procedure were selected as the response factor (Y).

## 2.4. Toxicity

### 2.4.1. *Daphnia Magna*

Newborn daphnids (<24 h old) were exposed (24h or 48h) to samples according to the ISO 6341 method (2013). Daphnids were grown at 20  $\pm$  1  $^{\circ}$ C under a light source of 1000 lux using cool lamps and control tests were carried out at the same temperature without light emission. They were fed with *P. subcapitata* (300,000 cells ml<sup>-1</sup>). In each quadruplicate treatment schedule, 20 daphnids were scored according to their immobilization frequency in 10 ml sample volume.

Each assay was considered valid only when the immobilization in the control solution was less than or equal to 10%.

### 2.4.2. *Pseudokirchneriella subcapitata*

The algal growth inhibition test of *Pseudokirchneriella subcapitata* was carried out according to the ISO 8692 (2012). Cultures were kept in Erlenmeyer flasks at the same conditions of *D. magna*. The toxicity tests were initiated from an algal concentration of 10000 cells ml<sup>-1</sup> and conducted in three replicates using 25 ml sample volume. The endpoint consisted of cell growth inhibition, which was measured after 72 h in a Burkler cell counting chamber and calculated by dividing the difference of the number of control and sample cells to the number of control cells.

### 2.4.3. *Lactuca Sativa*

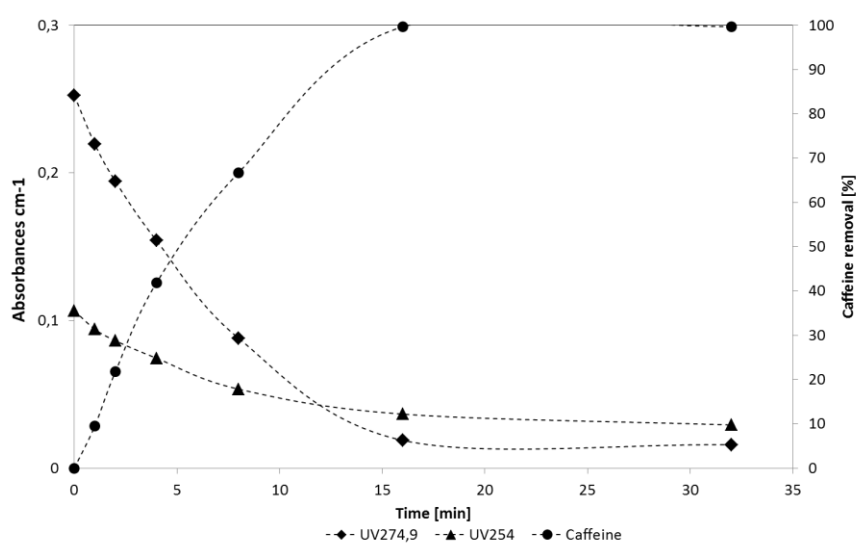
The phytoassay evaluated the possible sample toxicity by comparing the values of germination and root elongation of seeds with those obtained for seeds placed in controlled conditions. The test with *L. sativa* used Petri dishes (50 mm diameter) with one sheet of Whatman No. 1 filter paper as support. Germination and growth experiments were conducted in aqueous solutions at controlled pH, in three replicate experiments. In parallel a series of control experiments was performed. After the addition of 15 seeds and 4 ml of test solutions, the Petri dishes were sealed with parafilm to ensure closed-system

models. The seeds were placed in a growth chamber at 25 °C. After 5 days, the number of seeds germinated was counted and the radical length was measured.

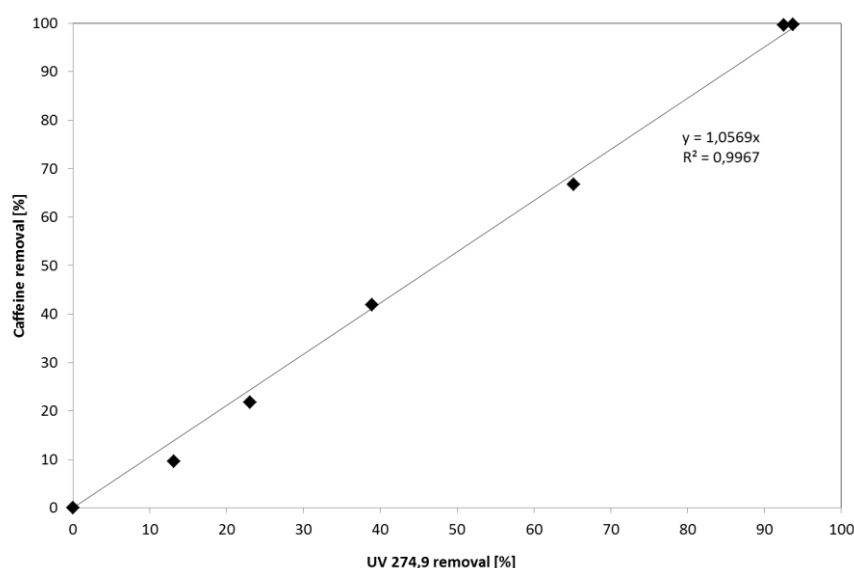
### 3. Results and Discussion

#### 3.1. Preliminary experiments

Preliminary experiments at 5 mg l<sup>-1</sup> of caffeine and 0.1 g TiO<sub>2</sub> l<sup>-1</sup>, room temperature and original pH were carried out in order to evaluate the degradation rate of caffeine. The disappearance of caffeine by photocatalysis as a function of irradiation time is shown in Figure 2a, where it can be perceived that almost total removal was achieved within 15 min of irradiation. The plot of C versus t obtained in our study fits well with the pseudo first order model ( $R^2 > 0.99$ ) corresponding to photocatalytic degradation rate constant of 0.1399 min<sup>-1</sup>.



(a)



(b)

**Figure 2.** a) Photocatalytic degradation of 5 mg caffeine l<sup>-1</sup> and 0.1 g TiO<sub>2</sub> l<sup>-1</sup>  
b) UV-HPLC data correlation

As shown in Figure 2b the removal trend of UV<sub>274</sub> was consistent with that of caffeine removal as measured by HPLC ( $R^2 = 0.9967$ ). Therefore changes in the concentration of caffeine were monitored using UV-Vis spectrophotometer in the subsequent experiments.

On the other hand, the formation of organic intermediates at wavelengths close to 254 nm (Figure 2a), could be observed during photocatalytic treatment. After 30 min of irradiation, UV<sub>254</sub> removal was only 70% which indicated that the oxidation of by-products required time to be completed. These results clearly suggest that although caffeine is almost fully consumed, its mineralization leading to CO<sub>2</sub>, H<sub>2</sub>O, and NH<sub>3</sub> is rather slow under TiO<sub>2</sub>/UV oxidative conditions. Therefore, degradation of caffeine is likely to generate persistent organic intermediates that are not so efficiently oxidized as compared to caffeine. Similar results were reported by Dalmazio *et al.* 2005 which observed that whereas 90% of caffeine at 31 mg l<sup>-1</sup> was degraded after 150 min of exposure to the TiO<sub>2</sub>/UV system (0.1 g TiO<sub>2</sub> l<sup>-1</sup>), only 13% of TOC removal could be observed.

### 3.2. Construction of polynomial expression and 3D response surface

The use of factorial design yields optimized parameters from a minimum set of experiments. Using codified values of the variables under study it is possible to obtain a polynomial expression that empirically describes the reaction yield (Brereton, 2003; Calza *et al.*, 2006). In order to evaluate the toxicity of by-products formed during the oxidation process, the oxidation time was set at 5 min in consideration of the preliminary study on degradation kinetic to avoid the complete mineralization of the caffeine.

Table 1 shows the experimental results (11 experiments performed, 2 variables, 2 levels for the 2<sup>2</sup> factorial design) for the response factor (Y) corresponding to the caffeine residual concentration (%) after 5 min of light irradiation, varying the concentration of TiO<sub>2</sub> and the initial caffeine concentration in a defined range.

Since the different input variables presented different dimensional units, these variables can only be compared if they are normalized to a common unit. Therefore, coded variables were a need during the modelling procedure.

The coded values taken for the calculations appear in Table 1 in parenthesis for TiO<sub>2</sub> concentration ( $x_1$ ), and initial caffeine concentration ( $x_2$ ). Only in this way, the input variables can be used in the single polynomial expression in Eq. (1).

The third column in Table 1 presents the experimental values obtained for the response factor (Y) and the fourth column presents the calculated values by way of the modelling procedure. Solving the matrix the following second grade polynomial was obtained:

$$Y = 32.065 + 4.068 x_1 - 20.633 x_2 - 1.681 x_1^2 + 9.239 x_2^2 - 3.705 x_1 x_2 \quad (1)$$

Taking into consideration only the first-order effect in Eq. (1), the optimum conditions for the photocatalytic degradation of caffeine seemed to be when TiO<sub>2</sub> ( $x_1$ ) is high and initial caffeine concentration ( $x_2$ ) is low. Moreover it seems that caffeine concentration affect likely the photocatalytic degradation displaying a high coefficient. A more important quadratic effect can be ascribed to the caffeine concentration, whereas a low quadratic effect is associated with the initial TiO<sub>2</sub> concentration.

The values obtained by the model ( $Y_{\text{calc}}$ , Table 1, last column) were compared with those of experimental data ( $Y_{\text{exp}}$ , Table 1, third column). These values are very close indicating a correspondence between the mathematical model and experiment ( $p < 0.001$ ).

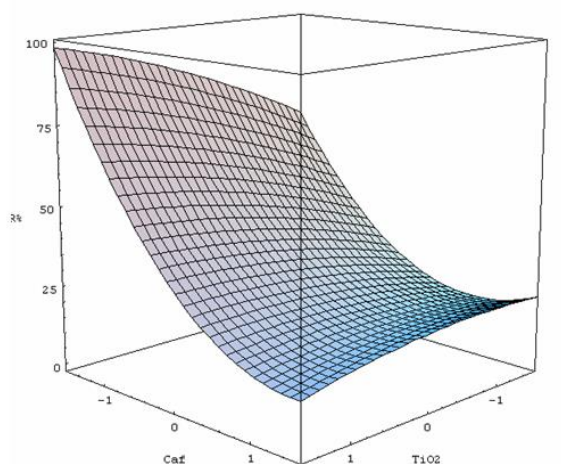
This mathematical expression describes the photocatalytic degradation of caffeine under given conditions which are not valid for a different type of pollutant or different set of conditions. The coefficients in the polynomial represent the weight of each variable ( $x_1$ ,  $x_2$ ) – corresponding to TiO<sub>2</sub> concentration and initial caffeine concentration, respectively – and the interaction between them.

Figure 3 depicts a 3D representation of the polynomial obtained from the matrix. The coordinates of the graph show the TiO<sub>2</sub> amount and initial caffeine concentration. The vertical axis represents the caffeine removal (%) after 5 min of light irradiation during the photocatalytic process.

**Table 1.** Experimental results from the factorial design of caffeine photocatalytic degradation

TiO <sub>2</sub> initial concentration $x_1$ (mg l <sup>-1</sup> )	Caffeine initial concentration $x_2$ (mg l <sup>-1</sup> )	Caffeine residual concentration $Y_{exp}(\%)$	Caffeine residual concentration $Y_{calc}(\%)$
50 (-1)	2 (-1)	46.99	51.73
50 (-1)	8 (1)	18.13	18.98
150 (1)	2 (-1)	63.93	66.99
150 (1)	8 (1)	20.26	19.42
29.3 (-1.414)	5 (0)	26.24	23.38
170.7 (1.414)	5 (0)	35.76	35.15
100 (0)	0.76 (-1.414)	85.56	81.22
100 (0)	9.24 (1.414)	20.12	20.98
100 (0)	5 (0)	30.67	32.03
100 (0)	5 (0)	31.18	32.03
100 (0)	5 (0)	35.10	32.03
100 (0)	5 (0)	31.47	32.03
100 (0)	5 (0)	30.70	32.03
100 (0)	5 (0)	33.26	32.03

According to our results the removal efficiency of caffeine gradually decreased with both TiO<sub>2</sub> concentration and caffeine initial concentration increasing, as well as increased under the higher level of TiO<sub>2</sub> concentration and the lower caffeine initial concentration. At low initial caffeine concentrations an increase of TiO<sub>2</sub> produced an even a minimal increase of removal efficiency. Conversely when the caffeine concentration increased, an increase of TiO<sub>2</sub> corresponded to a decrease of removal efficiency.



**Figure 3.** Response surface showing the effect of TiO<sub>2</sub> and initial caffeine concentration on the degradation rate expressed removal (%) after 5 min irradiation

This lead to conclusion that the caffeine degradation rate significantly decreased when  $\text{TiO}_2$  concentration and caffeine initial concentrations are too high.

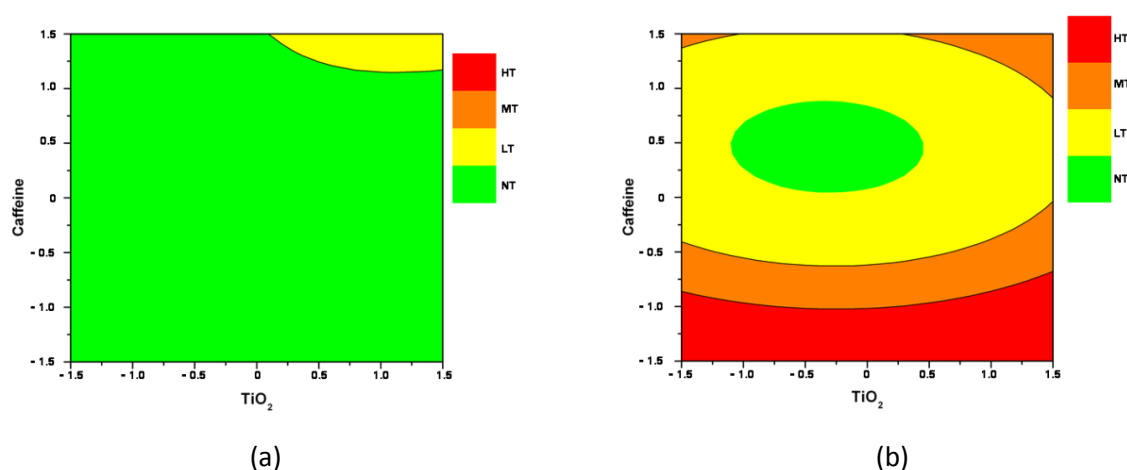
According to Figure 3 it can be expected that a similar trend could be observed also at high  $\text{TiO}_2$  concentration and low caffeine initial concentrations. In this case, the effect can be attributed to the relevant scattering of incident light (Calza *et al.*, 2006).

### 3.3. Toxicity

In order to compare chemical and ecotoxicological results, toxicity data were organized following the same scheme of the chemical ones. Finally results were represented in bi-dimensional graphics to better highlight the toxicity regions corresponding to different initial  $\text{TiO}_2$  and caffeine concentrations. According to the Italian Institute of Environmental Protection and Research (ISPRA, 2012), four intervals were adopted to define *D. magna* toxicity regions.

For the assays at 24 h only two kind of regions, corresponding to no toxicity (NT) and low toxicity (LT) area, could be identified as shown in Figure 4a. The smaller ones, LT, was found in correspondence of medium-high  $\text{TiO}_2$  values and high initial caffeine concentration. This result could be explained by the presence of toxic intermediates produced by the partial oxidation of the caffeine (25% removal, Figure 2). At this concentrations, the amounts of  $\text{TiO}_2$  could have not been enough to degrade the by-products which resulted toxic to the crustaceans. No toxicity was detected for the others values of  $\text{TiO}_2$  and initial caffeine concentrations investigated.

As expected toxicity on *D. magna* at 48 h (Figure 4b) displayed a different pattern than at 24 h. In this case only a restrict NT region could be identified in correspondence of low-medium  $\text{TiO}_2$  concentrations and medium-high concentration of initial caffeine. As consequence an increase of toxicity was detected in the contiguous areas. Independently from the  $\text{TiO}_2$  concentration at low initial caffeine concentration, after 5 min of oxidation, in correspondence of maximum caffeine removal, *D. magna* at 48 h showed a high toxicity which could be attributed to the presence of derivatives. This finding confirms that caffeine is quickly degraded, but not mineralized as quickly, and that persistent toxic organic intermediates resist further oxidation. Similar results were observed in an our previous study (Lofrano *et al.*, 2014) where the detoxification of vancomycin by products was monitored, and reported in studies dealing with diclofenac photocatalytic oxidation, as well (Rizzo *et al.*, 2009).



**Figure 4.** Toxicity (*D. magna* after 24 and 48 h of exposure time). Values are the mean of four replicates. No toxic: NT<20%; Low toxic: 20%<LT<50%; Medium toxic: 50%<MT<80%; High toxic: HT>80%

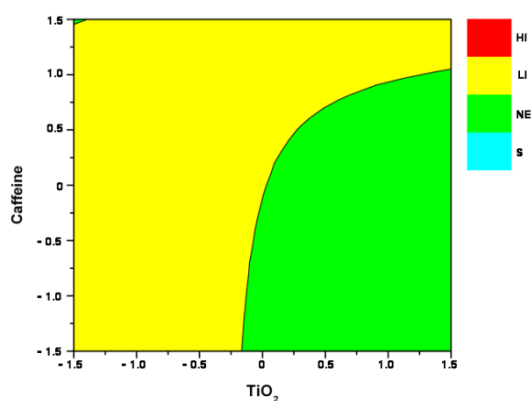
The formation pathways of intermediates during the photocatalytic degradation of caffeine have been widely investigated (Dalmazio *et al.*, 2005; Chuang *et al.*, 2011). In the studies of Dalmazio *et al.*, 2005 the ESI mass spectrum at zero irradiation time detected an intense ion of  $m/z$  195 corresponding to protonated caffeine. After irradiation for 90 min as well as 150 min, ESI showed two additional and



increasingly abundant ions of  $m/z$  143 and 175. The ion of  $m/z$  143 indicated mass reduction of 52 Da (from caffeine of 194 Da) and is likely to be the protonated form of dimethylparabanic acid a known oxidation product of caffeine. The other ion of  $m/z$  175 indicated a mass increase of 32 Da from the protonated form of dimethylparabanic acid (incorporation of two oxygens) and therefore this intermediate was further oxidized likely at both of its *N*-methyl groups yielding di(hydroxymethyl)parabanic acid, detected in its protonated form of  $m/z$  175. In our case it can be assumed that after 5 min of oxidation the amount of by-products could be differently produced, in consideration of the different amount of TiO<sub>2</sub> and caffeine used.

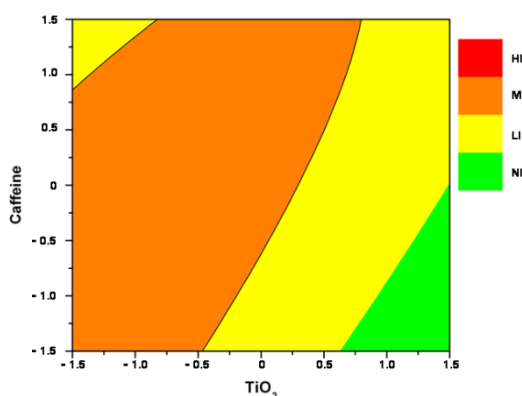
Literature data rarely provide qualitative and quantitative information on fate and effects of these active substances and their derivatives from biotic or abiotic transformations, that may make by-products more harmful than parent compounds (Isidori *et al.*, 2005; Lofrano *et al.*, 2014).

In order to define inhibition regions for *L. sativa* the scale proposed by ENEA (2003) was adopted, whereas for *P. subcapitata* four intervals were established based on dichotomus scale proposed by Balzamo *et al.* (2010). No effects or inhibition could be observed on *L. sativa* and *P. subcapitata* in correspondence of medium-high values of TiO<sub>2</sub> and low-medium initial caffeine concentrations, as shown in Figures 5 and 6 respectively.



**Figure 5.** Effects of photocatalytic degradation products on growth of *L.Sativa*.

Values are the mean (three replicates) of number of cells per millilitre in each treatment. Stimulation:  $S > 120\%$ ; No effects:  $80\% < NE < 120\%$ ; Low inhibition:  $40\% < LI < 80\%$ ; High inhibition:  $HI < 40\%$ .



**Figure 6.** Effects of photocatalytic degradation products on growth of *P. subcapitata*.

Values are the mean (three replicates) of number of cells per millilitre in each treatment. No inhibition:  $NI < 20\%$ ; Low inhibition:  $20\% < LI < 50\%$ ; Medium inhibition:  $50\% < MI < 80\%$ ; High inhibition:  $HT > 80\%$

On the other hand a greater inhibition area (MI) was associated to *P. subcapitata* respect to *L. sativa* where only a low inhibition (LI) was observed in correspondence of low medium values of TiO<sub>2</sub> and almost all caffeine concentrations investigated. Although *P. subcapitata* showed to be more sensitive to by-products than *L. sativa*, in both cases no high inhibition regions could be identified. These results were in agreement with a recent study of Zarrelli *et al.* (2014) where the toxicological effects of caffeine derivatives, obtained by a reaction between caffeine and sodium hypochlorite mimicking the chlorination step, on the freshwater rotifer *B.Calyciflorus* and the microalga *P.Subcapitata* were elucidated. Chronic exposure to these compounds caused inhibition of growth population on the rotifer while the algae seemed to be unaffected.

The chronic assays seems to be more appropriate than acute ones to detect the impact of by-products formed during the photocatalytic treatment of drugs (Isidori *et al.*, 2005; Lofrano *et al.*, 2014).

#### 4. Conclusions

In the present study the variation of both catalyst and caffeine concentrations in the photocatalysis process were evaluated through the use of response surface methodology (RSM) and the most favourable conditions in obtaining the drug abatement were established. In parallel ecotoxicity measurements on the treated solutions emphasized the need of evaluating the efficiency of the photocatalytic treatment taking into account detoxification process. In the regions corresponding to an increasing removal of caffeine, an increase in toxicity related to the formation of persistent by-products, which take time to be further oxidized, was observed.

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