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Evaluation of the aquatic toxic effect varied during the degradation of capecitabine under the environmental abiotic and biotic processes

Ruixin Guo, Fengzhu Zheng and Jianqiu Chen*

Department of Environmental Science, China Pharmaceutical University

Corresponding author:

Jianqiu Chen: Department of Environmental Science, China Pharmaceutical University, 210009, Nanjing, China, Tel.: + 86 25 86185190, Fax: + 86 25 86185190.

E-mail address: cjqalga@163.com

Abstract

The environmental risk due to the growing use of anticancer drugs has drawn wide public concern. The present study investigates whether the degradation of CAP under an abiotic process (under the UV irradiation) and two biotic processes (the action of the green algae and sludge) occurred and evaluate the aquatic toxicity of CAP during the environmental process. Our result indicated that CAP was completely degraded within 20 min after the UV irradiation with no significant change in the content of the total organic carbon (TOC). Aquatic toxicity assessment indicated that the toxicity increased if CAP underwent the UV irradiation process. In addition, CAP was persistent to the action of the green algae and the sludge, while the toxicity of CAP decreased after the biotic process, in which attributed to the action of the sludge. The green alga did not play the crucial role in the detoxification.

Keywords: Capecitabine, UV irradiation, biotic process, aquatic toxicity test

Introduction

Pharmaceutical residuals have attracted public and scientific concerns since they were first detected in the environment in the 1970s [1]. The chemotherapeutics, such as cytostatic, cytotoxic and antineoplastic drugs, are used to inhibit the growth and development of the tumor cells. Investigations of pharmaceutical consumption reveal a continue increasing in the use of anticancer drugs in recent years, resulting in an increased emission into the environment [2,3]. Many researches have focused on the analytics, elucidated environmental degradation, fate, and concentrations, investigated the ecotoxicological effects, and assessed the environmental risks of the pharmaceuticals, especially the cytostatic cancer medicines [3]. Generally, hospital effluents are directly discharged into the public sewage system without the pretreated process in most countries, which are more likely to carry potential ecotoxicology. Thus, hospital effluents are considered as a significant source of anticancer drugs in the aquatic environment due to the excretion of the patients on chemotherapy. The cytostatic pharmaceuticals, such as 5-fluorouracil, cisplatin, doxorubicin and etoposide, have been detected in the hospital effluent [4-6]. One thing worth noting is that, the increasing number of cancer patients could receive drug therapy by oral administration at home, and about 75% of cancer patients can leave for home after receiving the treatment of infusion or injection at hospital [7, 8]. Thus the domestic sewage is becoming another important source of anticancer compounds as well.

Benefited from many advanced methods in the detection of anticancer drugs [9], for now, many anticancer agents have been detected in the aquatic systems, like

5-fluorouracil (5-FU), ifosfamide, and cyclophosphamide [9], and several new compounds, such as imatinib mesylate (IM), and temozolomide, and capecitabine (CAP) [2, 10]. CAP is a new oral anticancer drug, playing their role by converting to the active compound 5-FU in vivo. 5-FU and its pro-drug CAP are pyrimidine analogues characterized as antimetabolites. This class of drugs inhibits DNA polymerase and induces cell cycle arrest and apoptosis. Because of the better clinical curative effect and higher security, CAP is popular with the cancer patients [11] and has been detected as a new compound in environmental samples, which has expanded the list of anticancer drugs measured in environment [12]. Based on therapeutical function and on the biological mode of action, certain groups of anticancer drugs are suspected to cause damage to key organisms in ecosystems. Thus, the environmental risk assessment for CAP in Europe has been considered and experienced [3].

As primary producers, microalgae are the key component of the aquatic ecosystems. They produce oxygen and organic substances, which were provided as food for other organisms, including invertebrates and fish [13]. The anthropogenic chemical effects on algae could directly influence the structure and function of the ecosystem, resulting in oxygen depletion and decreased the primary productivity[14, 15]. Additionally, algae is sensitive to most contaminants, even if at a relatively low concentration [16]. Therefore, algae is often applied to evaluate the toxic effects of the hazardous chemicals. Rotifers are another critical elements in the oceanic and freshwater levels food webs, linking the primary producers like the algae and higher trophic levels such as crab, shrimp and fish [17]. Because of their important ecological

roles, rotifers are also widely used to be the ideal biological test models for evolutionary ecology and evolution of sex [18], population dynamics, [19] aquatic ecology [20, 21] and chemical communication [22]. Due to the advantage characteristics like small size, sensitivity to vast number of toxic substances, easy lab-culture and cost-effective, rapid population growth rates and high population density in a short time [17], rotifers as the test organism are consequently well applied.

Considering that the anticancer agents have been detected in superficial waters, representing an environmental risk to the biota, it is necessary to emphasize that the ecotoxicological implications of these substances have been insufficiently studied in aquatic organisms. The current study investigated the toxicity of six cytostatics on the common freshwater rotifer *Brachionus calyciflorus* [23]. However, It also suggests that the phototransformation effect of the pharmaceuticals is a main factor in considering their degradation when they exposed to environment [24]. Several researchers reported that pharmaceuticals were easily photodegraded. For example, more than 50% of the anti-inflammatory drug diclofenac was degraded after a 4 h UV irradiation [25], and only half concentration of the anticancer drugs 5-FU was residual after a 15 min UV irradiation [9]. The degradation of CAP is primarily through microbial transformation and/or photochemical processes, which could lead to partial degradation and the accumulation of some hazardous products in the environment. Thus, if CAP is dispersed in the aquatic environment, not only a given proportion of it impacts the aquatic organisms directly, but the compounds which might undergo the photochemical reactions might also harm the organisms. Therefore, evaluating how

the aquatic toxic effect varied during the degradation of CAP in the environment is meaningful. The daphnid and algal toxicity test reported before [3], nevertheless, the coverage of ecological impacts of the pharmaceutical compound, especially during the environmental abiotic and biotic processes, on the aquatic organisms was rather sparse. Thus, the aim of the study was to investigate whether the degradation of CAP under an abiotic process (under the UV irradiation) and two biotic processes (the action of the green algae and the sludge) occurred and evaluate how the toxicity of CAP varied during the environmental processes. It should to better to understand the ecological risk when CAP arrives into the aquatic environment.

Materials and methods

Test compound and analytical methods

The anticancer capecitabine (CAP, CAS NO.: 154361-50-9) was provided by Nanjing Sanhome pharmaceutical company. Methanol and acetonitrile were HPLC grade obtained from Merck & Co Inc. (Germany). The concentration of CAP was analyzed by HPLC, coupled with a C18 reversed phase column at 30℃. The mobile phase had methanol-acetonitrile-0.1% acetic acid at a flow rate 1.0 mL min^{-1} [26].

Test organisms

The selection of the algae species and the rotifer species as a representative aquatic organism in this study was justified by its environmental abundance and role in several ecological processes in freshwater communities. The strain of the green algae *Chlorella pyrenoidosa* was obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences. The algal cells were incubated with BG-11 medium [27]

and maintained at 25 ± 1°C under an illumination intensity of 2000 lux, with a 12 h/12 h light/dark interval. The freshwater rotifer *Brachionus calyciflorus* was originally isolated from a pond on the Jiangning campus of China Pharmaceutical University. Cultures were established as a clone from a single female and maintained in our laboratory for 3 months. The rotifer was cultured in the artificial freshwater medium (EPA medium, containing $NaHCO_3$ 96 mg, $MgSO_4$ 60 mg, $CaSO_4$ 60 mg, KCl 4 mg in 1 L distilled water with the pH adjusted to 7.5) with the green algae *C. pyrenoidosa* as the diet. The test animals were maintained at 25 ± 1 °C on the photoperiod 12: 12 (L: D) with 2000 lx light. To build an optimal growth environment for the rotifer, the medium and food should be renewed at daily regular intervals. The sludge was aerated cultured the lab before the experiment at 25 ± 1 °C.

Experimental set-up

With the aim of the present study, all the experiments were performed in two parts: In part I, whether the concentration change of CAP was evaluated when the compound underwent the individual UV irradiation process, the action of the green algae and the sludge (see Fig. 1). Quartz photochemical immersion well reactor and high pressure mercury lamps of 500 w emitting 365 nm were used for the UV irradiation process. A CAP solution of 20 mg L^{-1} was added into the photochemical reactor with the UV irradiation. Thus, three concentrations of CAP has been considered: CAP at 20 mg L^{-1}, CAP with the concentration declined from 20 mg L^{-1} to 10 and 0 mg L^{-1}, respectively, when the compound underwent the UV irradiation process. In the algal action process, a fresh culture medium of 200 mL was added which mixed with the green algae and

CAP. The initial algal density and the concentration of CAP were 10.0×10^6 cells mL^{-1} and 20 mg L^{-1}, respectively. In the sludge action process, CAP at the given concentration was mixed with the sludge in a 1 L glass bottle, in which the final mass of the sludge was 1.4 g L^{-1}. Samples were taken at intervals to determine the residual concentration of CAP by HPLC. The total organic carbon content (TOC) of the samples was also measured by TOC analyzer (Shimazdu TOC-L analyzer). We also evaluated the concentration change of CAP after a combined algae-sludge action process and a combined UV-algae-sludge action process. The above three concentration conditions were also considered in the combined process. The temperature and pH value of the two parts was set at 25 ± 1 °C and 7.5, respectively.

The experiment in part II was applied for the aquatic toxicity assessment. In this study, two aquatic organism species, the green algae *C. pyrenoidosea* and the rotifer *B. calyciflorus* were chosen to evaluate the toxicity of CAP and how the toxicity changed when CAP underwent the chemical and biological processes. All treatments were cultivated as the cultural condition of the green algae. The temperature was set at 25 ± 1 °C. Samples were taken from the culture vessels at 72 h, measuring the OD value at 680 nm. The toxicity bioassay is based on the population growth inhibition of *C. pyrenoidosea* caused by the presence of CAP and the compound which underwent the UV irradiation process. The population growth rate (r) was calculated from the formula: $r = (\ln N_t - \ln N_0)/t$; where N_t and N_0 are population sizes at day 0 and day t, and t is the sampling time [28]. The inhibition rate (IR) was calculated according to r: $IR=1-r_t/r_c$; where r_t and r_c is the algal population growth rate in the treatment group

and the control. The initial algal density was 1×10^6 cells per milliliter when the green algae was mixed with the testing solutions. The algal culture with BG-11 was used as a control. Each experiment had three replications per treatment.

The toxicity test of the above solutions on *B. calyciflorus* was performed on the basis of the standard guidelines [29]. For each test, 10 juveniles (less than 24 h old) per hole were exposed to the toxic solutions (CAP under the different processes). 24-well culture plates were incubated in the continuous darkness at 25±1°C for 24 h, no feeding during the experiment. Rotifers which were cultured in EPA medium without testing solutions was applied as a control. After 12 and 24 h of the exposure, the number of the dead rotifers was determined as mortality to compare with control. Rotifers were considered dead if there was completely motionless of the mastax and cilia over a period of 30 s [30]. The rotifer was also applied to evaluate whether the toxicity changed when CAP underwent the action process of the alga and the sludge. Six replicates were set for each sample.

Statistical analyses

SPSS Statistics 19.0 was used for data analyses. The difference test results were analyzed statistically with one-way analysis of variance (one-way ANOVA) and multiple comparisons (LSD test) with significance set at $p<0.05$. All the figures were produced using Sigmaplot version 12.5.

Results

The degradation of CAP under the abiotic and biotic processes

The concentration of CAP at 20 mg L^{-1} as the initial concentration under the UV

irradiation was investigated and the results are shown in Fig.2. The concentration change of CAP was corresponded to a first order kinetic:

$$C = C_0 \, e^{-kt}$$

where C was the residual concentration of CAP at the irradiation time t, C_0 is the initial concentration of CAP and k is the rate constant. The photolysis rate constant was 0.5012 min^{-1} (R=0.9999), and the half-time was 1.4 min in this model. According to the photolysis equation, 99.9% of the parent compound were removed within 12 min, which indicated that the degradation of CAP under the UV irradiation was a fast process. With respect to the results of TOC in Fig.2, there was no significant change when CAP underwent the UV irradiation process.

In the present study, however, the results in Fig.3 showed that CAP was persistent to the action of the green algae. Only 0.64% of CAP were eliminated after the action of the algae at the end of 6 h (see Fig.3 A). In addition, our present results also indicated that most of CAP was detected after a 6 h sludge action process (see Fig.3 A). Although the combined action of UV, the algae and the sludge on the target drug has also been considered, only 6.73% of CAP disappeared after the combined biological action, in which the relevant main removal efficiency attributed to the sludge (see Fig.3 B). It is also demonstrated that UV irradiation could not further improve the biodegradation of CAP and only the UV irradiation play the dominant role in the combined UV-algae-sludge action process.

The aquatic toxic effect evaluation

As shown in Fig.4, after an exposure of 72 h to 20 mg L^{-1} of CAP, the algal

growth inhibition rate was only 2.5%, indicating that the target compound showed no impact on the algal growth. However, the algal growth inhibition rate increased 14 times when the residue of CAP was 10 mg L^{-1} after the UV irradiation process. Even if CAP was photodegradated completely by the UV (the final concentration was 0 mg L^{-1}), it also caused 25.7% of the inhibition rate of the algal growth. The result indicated that CAP could be well degraded after the UV irradiation process in a short time, while the toxicity to the algae was dramatically increased significantly (p<0.01).

The rotifer *B. calyciflorus* showed a mortality of 15% when exposed to the parent substance at 20 mg L^{-1}, while there was no rotifer died in the control. Additionally, CAP at the two different concentrations after the UV irradiation process caused the mortality of the rotifers arrived to about 65%, which increased 4.3 times by that when the rotifers were exposed to the parent compound directly. Statistical analysis showed that the chemical action process had a significant influence on the toxic effect of CAP to the rotifers (p<0.01), and the rotifer was more sensitive than the algae not only to CAP but also to the presence of the photolysis products. When the concentration of CAP decreased from 20 to 10 mg L^{-1} by UV irradiation and then underwent the action of the algae, it also caused nearly 65% of the test rotifers died after 24 h, which was significantly higher than that when the rotifers were exposed to the target compound (p<0.01). Whether the compound underwent the same abiotic process with an individual action of the sludge and a combined action of the algae and the sludge, only 6.67% and 5.00% of the test rotifers died during the same time, respectively. In addition, if CAP was completely photodegraded after the UV

irradiation process, it caused higher than 60% of the test rotifers died, while the mortality fell to 56.67% and 11.67% when the compound underwent the subsequent action of the algae and sludge, respectively. What is more, a relevant low mortality (3.3%) was observed when the compound underwent the combined action of the algae and sludge.

Discussion

Generally, most of organic pollutions could be disintegrated structurally and even completely mineralized under the UV irradiation [31]. Our present results, however, showed that despite the removal rate of CAP reached up to 50 %, there was no significant change in the content of the TOC. The high removal rate with low mineralization degree demonstrated that the target anticancer drug was decomposed into small molecular weight organic matters rather than carbon dioxide, water or inorganic salts directly. The previous chemical analyzes showed that CAP was disintegrated to various transformation products if under the UV irradiation, such as $C_{10}H_{15}N_3O_3$, $C_{15}H_{24}O_7F$ and $C_{15}H_{23}N_3O_7$ [9], which also support our results. Similarly, although 65% of mitoxantrone, another common used anticancer drug, was degraded after a 140 min UV irradiation, the value of TOC was almost unchanged with the direct photolysis even if the UV radiation time was extended to 2 h [32].

Microalgae, bacteria and protozoa are the critical components in the aquatic environment. Several studies have shown that microalgae play a considerable role to accumulate and remove environmental contaminations, such as heavy metals, pesticides and antibiotics [33 34, 35]. However, our results indicated that compared to the

relevant faster degradation by UV, the green algae was not able to degrade the target compound directly. On the other hand, the adsorption and the bio-removal of the sludge are usually considered as the major elimination mechanisms for most organic pollutants, especially in the biological process of the sewage treatment systems. As an important medium in the water environment, the sludge is indispensable on the migration and transformation of the organic contaminants. Our present study also aimed to reveal whether the sludge could removal CAP effectively. Most of CAP was residual after 6 h, which means that CAP was also persistent to the action of the sludge.

Algae and rotifers are useful as a model in ecotoxicology because they play an important role in the aquatic system and show more sensitivity to most organic pollutions. Some contaminants in water are known to exert disadvantageous effects on algae and rotifer. The impact of the target organic compounds is usual species-dependent. In the present study, the algal population growth inhibition rate was only 2.50% in average at a concentration of 20 mg L^{-1}, which implied that the green algae species was not sensitive to the impact of CAP. In contrast, CAP was strongly toxic to the algae *Selenastrum capricornutum* with a growth-rate EC_{50} of 2.0 mg L^{-1} [36]. Additionally, although the aquatic toxicity of CAP on rotifers has been reported [23], the assessment of the ecotoxicological effects when the target compound underwent the chemical or biological action has been limited. UV irradiation is an efficient method for removing pharmaceuticals, certainly for CAP. Many previous studies also pointed out that the photo irradiation process achieved a lower

mineralization degree of the target compounds, while the reaction products were usually higher toxic than the parent compounds [25, 37, 38, 39]. Thus, it is necessary to evaluate whether the toxicity changed after the photo-irradiation process. In the present study, the toxicity testing was expressed in the population growth inhibition rate of *C. pyrenoidosa* and the mortality of *B. calyciflorus*. For the green algae, compared with the parent compound, a significant impact on the algal population growth occurred when half of CAP was degraded by UV (10 mg L^{-1} residue). We have also observed a significant inhibition of the algal population growth even if the target compound was degraded nearly completely (0 mg L^{-1} residue). On the other hand, with respect to the results in Fig.5, there were significant differences in the mortality between the rotifers were exposed to CAP itself and to the compound under the UV irradiation (p<0.05). Compared with the impact of CAP, more rotifers died when half of the target compound was degraded under UV irradiation. Thus, with respect to the changing concentration of CAP in Fig.2, our results indicated that the disappearance of the initial substrate is not necessarily associated with a decrease in the toxic effect. It implied that CAP could be excreted as parent compound or as one or more metabolites and, once in the water, they can undergo a biotic transformation into different compounds that can be more persistent and more toxic than the parent compounds.

In addition, although most of CAP was persistent to the action of the algae and sludge, whether the toxicity changed by the bio-action also raised our concern. From Fig. 5, the mortality of the test rotifers decreased after the action of sludge no matter

how the concentrations changed (decreased to10 or 0 mg L^{-1}) after the UV irradiation, which indicated that the adverse effect weakened after the action of the sludge. 5-FU usually viewed as the metabolite of CAP, especially in the gastrointestinal tract. Thus, the previous toxicity testing has documented and compared the toxic effect of CAP and 5-FU. Generally, 5-FU was more toxic than CAP in the daphnia acute test, while less toxic impact on the algal growth-rate inhibition test[3]. CAP is administered orally, readily absorbed from the gastrointestinal tract, and converted to the 5-FU, therefore the metabolism of CAP to 5-FU proceeds rapidly and clearly. However, no further reports on the degradation process under the UV irradiation have been located. Our results indicated that the degradation of the parent compound by UV irradiation caused the increasing of the toxicity of the by-products, while the toxicity was controlled by the subsequent action of the sludge. It implied that compared with the parent compound, the by-products might be available by the sludge much easier. Thus, the decreased toxicity may be attributed to the surface sorption, intracellular sorption or intracellular metabolites. In addition, if the UV radiation time was extended, the by-products might be further degraded or mineralized completely. It might be a considerable process which also alter the final toxic effect of the target compound.

And, more remarkable, while the green algae did not play the crucial role in the detoxification individually, the combined action of the algae and the sludge could cause the rotifer mortality fell further to 7.50% and 3.33%, respectively. Compared with the contribution of the individual sludge on detoxification, a further detoxification for the rotifers attributed to the action of algae before the sludge in the

combined process. It implied that when exposed to the CAP and the by-products, the algae might secrete several possible metabolites such as polysaccharide, protein or other extracellular polymeric substance could produce a possible activation effect on the subsequent action of the sludge. The more advanced separation and analytical methods should be considered in our following study, which could help us to better deduce the chemical and biological reactions and decipher the mechanism.

Conclusion

In spite of nearly 100% of CAP was degraded after a 20 min of UV irradiation, the photolysis process was considered highly enhancing the toxicity for the green algae *C. pyrenoidosa* and the rotifer *B. calyciflorus*. In the combined abiotic and biotic process, UV irradiation was failed to improve the subsequent CAP biodegradability. However, the toxicity of CAP decreased after the biotic process, in which attributed to the action of the sludge. The existence of CAP in the environment had potential harm for the aquatic organism, especially underwent the abiotic process like UV irradiation, while the ecological impact could be lighten after the biotic process.

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Table 1 CAP under the abiotic and biotic processes

Group	The initial concentration	The finial concentration	Abiotic processes	Biotic processes	
			UV irradiation	alga	sludge
1	20	10	+	+	-
2		10	+	-	+
3		10	+	+	+
4		0	+	+	-
5		0	+	-	+
6		0	+	+	+

Concentration: mg L^{-1}

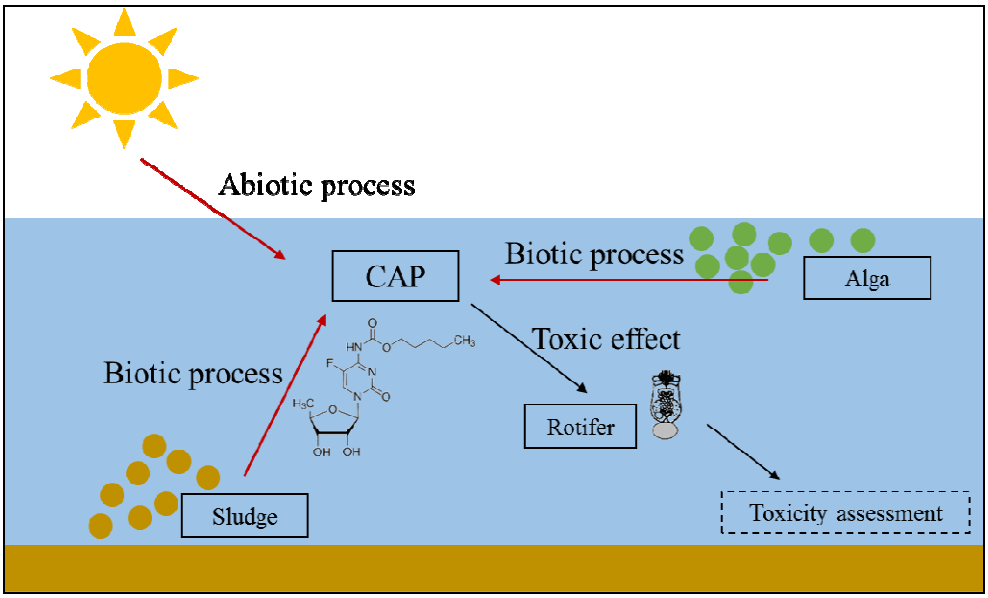

Fig.1 Experimental designschematic

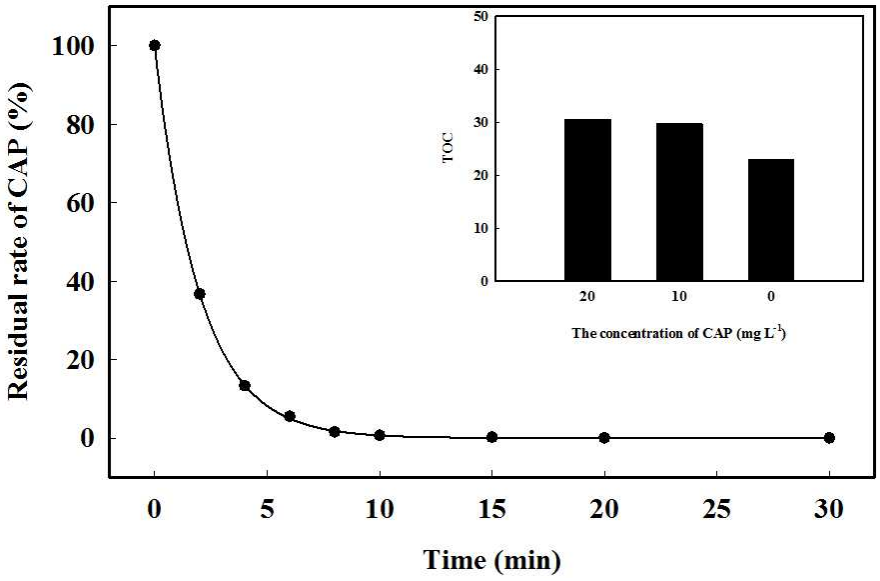

Fig.2 Kinetics of CAP elimination and the change of total organic carbon (TOC) when exposed to UV irradiation

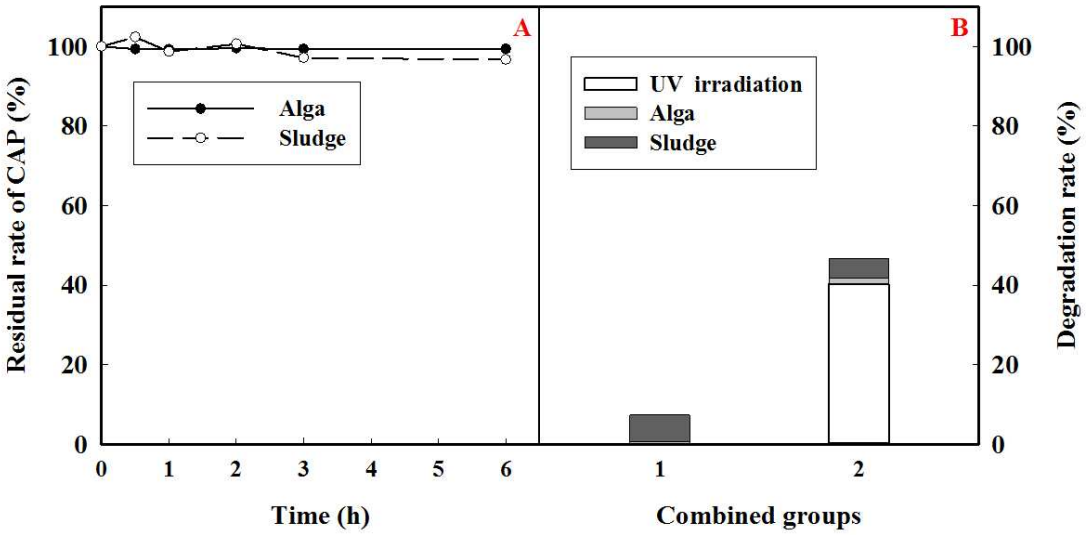

Fig.3 The concentration change of CAP during the different processes. A: under the individual action of the algae and the sludge, respectively; B: under the combined biotic process (algae + sludge) and the combined abiotic and biotic process (UV + algae +sludge).

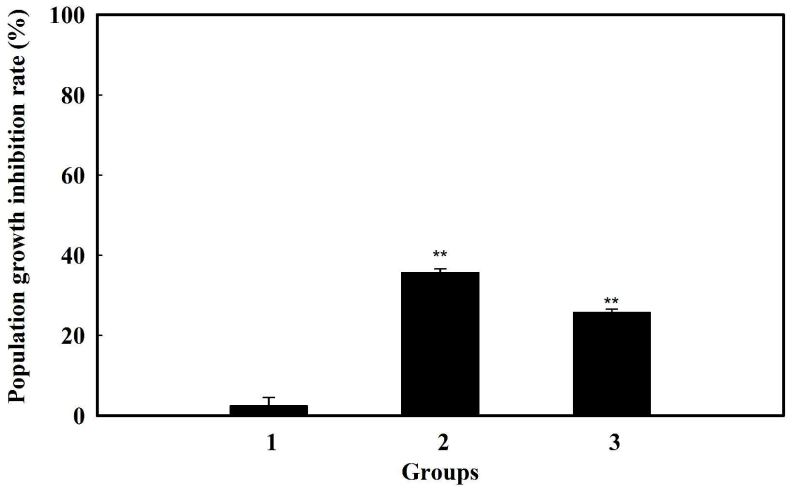

Fig.4 Algal toxicity bioassay of CAP during the different conditions. Group 1: CAP at 20 mg L^{-1}; Group 2: half of CAP was removed after the UV irradiation (10 mg L^{-1} residue); Group 3: All of CAP was removed after the UV irradiation (0 mg L^{-1} residue), (**: p<0.01).

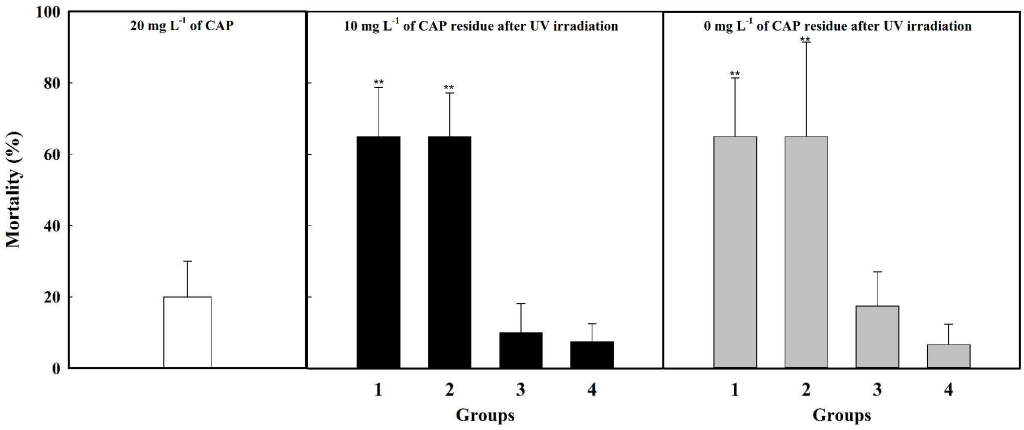

Fig.5 Rotifer toxicity bioassay of CAP at 20 mg L^{-1}, and 10 or 0 mg L^{-1} after the UV irradiation and different subsequent biotic process. Group 1: without any bio-action process; Group 2: under the individual action of the alga; Group 3: under the individual action of the sludge; Group 4: under the combined action of the alga and the sludge (**: p<0.01).