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Ecotoxicological analysis of fly ash and rice-straw black carbon on *Microcystis aeruginosa* using flow cytometry

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ABSTRACT

Black carbon (BC) has a strong affinity for hydrophobic organic compounds (HOCs), and it is a potential material to control HOCs pollution in aquatic ecosystems. Here, flow cytometry (FCM) was used to evaluate the ecotoxicological effect of fly ash, rice-straw ash, and their acid-demineralised products on the growth of *Microcystis aeruginosa*. It was found that the BCs had little negative effect on cyanobacteria, when the content of BCs was not above 1 mg ml^{-1}. However, higher doses of BCs (>2 mg ml^{-1}) had an obvious negative effect on cell density and esterase activity, especially for BCs with acid treatment, which greatly inhibited cell density caused by its high adsorptivity for cyanobacteria. The BCs had little impact on the fluorescence intensity, only with a slight stimulation in later period, so the fluorescence intensity was a less sensitive indicator than cell density and esterase activity. Considering ecotoxicological effect of BCs on the algae, the application concentration of BCs for HOCs pollution control as *in situ* remediation material would better not exceed 1 mg ml^{-1}.

1. Introduction

Black carbon (BC) is a highly aromatic polymer formed from the incomplete combustion of biomass and fossil fuels, and it represents a major organic component in aquatic sediments (Kwon and Pignatello, 2005). BC plays an important role in the migration and transformation of hydrophobic organic compounds (HOCs) in the environment because of its strong affinity to HOCs. In addition, BC has a long lifetime in the environment, owing to its chemical and thermodynamic stability (Brodowski et al., 2005; Forbes et al., 2006). With rich sources and minimal secondary contamination, BC is regarded as a potentially attractive *in situ* remediation material of HOCs pollution because of its high adsorptivity (Lou et al., 2011a, 2011b). It is therefore necessary to study the ecological influence of BC on aquatic environments.

Few studies have evaluated the toxicity of BCs. For example, Kobeticova et al. (2010) evaluated the effects of soil amended with incineration ash on earthworms. At the lowest dose, there was little or no influence of incineration ash on the weight of the earthworms, but at higher doses (25 percent and 50 percent), some decline in weight was found. Numerous studies revealed that the lower fly ash (FP) incorporated with soil could modify the physico-chemical, biological and nutritional quality of the soil. However, the higher dosage of FP incorporation resulted in heavy metal pollution and hindered the microbial activity (Adriano et al., 1978; Mittra et al., 2005; Pandey et al., 2009; Pandey and Singh, 2010). According to Singh et al. (2011a, 2011b), FP not only contains almost all the essential plant nutrients, but also could increase the availability of mineral nutrients for plant growth quality (Pandey and Singh, 2010). FP could improve the soil physico-chemical properties and fertility (Pandey and Singh, 2010), enhance soil microbial activities, and increase plant productivity when applied in combination with various organic manures (Singh et al., 2011a, 2011b). While several short-term laboratory incubation studies found that the addition of unweathered FP to sandy soils severely inhibited microbial respiration, numbers, size, enzyme activity and soil nitrogen cycling processes, such as nitrification and N mineralisation (Arthur et al., 1984; Garau et al., 1991; Pichtel and Hayes, 1990). Our research group had also used earthworms, as well as seed germination tests, to evaluate the toxicity of rice-straw ash (RP)-amended sediments. Those results indicated that compared with the control, high (ten percent) RP content caused a little but statistically significant ($p<0.01$) increase in DNA damage (Cui et al., 2009). These studies all focused on the toxicity of BCs in soil and were not adequate to examine the underlying mechanisms of the ecotoxicological effects of BCs in aquatic ecosystems.

Flow cytometry (FCM) is a useful tool for testing the toxicity of pollutants on both marine and freshwater microalgae (Franqueira et al., 2000). FCM allows for the rapid analysis of structural characteristics of cells without staining, such as cell volume and chlorophyll *a* autofluorescence (Franqueira et al., 2000). Esterase

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activity can also be rapidly determined when cells are stained with fluorescein diacetate (FDA) (Adams and Stauber, 2004). Compared to traditional growth-based inhibition assays, FCM provides the statistical result of more than 10,000 individual cells rather than a mean value tested at a population level (Rioboo et al., 2009), excluding the influence of individual difference efficiently in eco-toxicity assays (Xiao et al., 2010).

In our previous research, it was found that fly ash (FP), rice-straw ash (RP), and their acid demineralisation products, fly ash black carbon (FC) and rice-straw black carbon (RC), had high sorption capacities of HOCs. Furthermore, FC and RC had higher sorption capacity than their precursors (FP and RP). In this paper, the feasibility of BCs application for the control of HOC pollution in aquatic ecosystems was explored. To do so, FCM was used to evaluate the toxicity of BCs, and the effects of different concentrations of various BCs (FP, RP, FC and RC) on cell density, esterase activity, and in vivo chlorophyll *a* fluorescence of cyanobacteria were investigated.

2. Materials and methods

2.1. Preparation of RC and FC

Rice-straw ash (RP) and fly ash (FP) were used as precursors of BCs. Dry rice-straw was cut into small pieces (<20 mm) and oven-dried at 105 °C. The rice-straw was then placed in a ceramic pot, covered with a fitted lid, and pyrolysed under oxygen-limited conditions for 3 h at 600 °C (Chun et al., 2004). Fly ash produced during the combustion of coal (at 1400 °C) was collected on electrostatic filters at the Hangzhou Thermoelectric Plant, in Zhejiang Province, China. These samples were treated to get purified BC using acid demineralisation methods similar to those reported in previous studies (Chun et al., 2004; Haberstroh et al., 2006). Briefly, each BC sample (10 g) was treated in 200 ml of 2 M HCl for 24 h at 25 °C, and then centrifuged at 4800 rpm for 20 min to remove the supernatant; this procedure was repeated four times. Next, the same procedure was performed again, except that 1 M: 1 M HCl–HF solution was used instead of 2 M HCl (Luo et al., 2011). The treated samples were then thoroughly washed with distilled water five times to remove residual acids, Si, and soluble salts. The treated rice-straw charcoal and fly ash were oven-dried overnight at 105 °C, and labelled as RC and FC, respectively.

2.2. Algal cultures

The *Microcystis aeruginosa* FACHB 469 strain was obtained from the Freshwater Algae Culture of Hydrobiology (China). The cyanobacteria were grown in sterilised BG11 medium (Allen and Stanier, 1968) within 250-ml Erlenmeyer flasks. Flasks were placed in a growth chamber on a 12:12 h light/dark cycle at 70 μmol photons m^{-2} s^{-1}, and temperature of 28 °C. Cultures were not used until cyanobacteria were in an exponential growth phase.

2.3. Characteristics of RP, RC and FP, FC

The elemental composition (C, H, and N) of the BCs was determined by an Element Analyser (EA 1110, USA). The surface area and pore volume of the BCs were measured by a 100 CX surface area analyser (Coulter Omnisorp, USA). The Brunauer–Emmerr–Teller (BET) equation was used to calculate the surface area of the BCs, and the total pore volume (i.e., the sum of micro- and meso-pore volume) was defined as the volume of nitrogen adsorbed at the $P/P_0=0.981$ (Mui et al., 2010). The surface acidity and basicity were determined using Boehm's titration method (Boehm, 1994; Cai et al., 2009; Chun et al., 2004). Each kind of BC (0.500 g) was accurately weighed, and reacted with 25 ml of 0.1 M Na_2CO_3, $NaHCO_3$, NaOH, $NaOC_2H_5$, or HCl for 48 h in 50 ml conical flasks. The back-titration was carried out using 0.1 M HCl or NaOH to neutralise the excess acid or base. Surface acidity and basicity were calculated on the basis of the following assumptions: $NaHCO_3$ neutralises carboxyl groups only; Na_2CO_3 neutralises carboxyl and lactonic groups; NaOH neutralises carboxyl, lactonic, and phenolic groups; $NaOC_2H_5$ neutralises carboxyl, lactonic, phenolic, and quinonyl groups; and HCl neutralises all basic groups.

2.4. Determining cell density, esterase activity, and in vivo chlorophyll a fluorescence intensity

Each BC at four different doses were added (0.05, 0.1, 0.2, and 0.5 g), along with a control, to 100 ml of cyanobacteria solution. These values were consistent with our previous research regarding the adsorption of pentachlorophenol. Samples were taken at 4 h, 1 d, 2 d, 5 d, and 11 d after the introduction of the BCs.

FCM was performed to measure the metabolic activity and the percentage of viable cells. Cell samples were stained with 10 mg l^{-1} of FDA (Sigma, No. F7378). Healthy cells take up FDA and convert it to measurable fluorescence. This was detected in the FL1 detector (515–545 nm) and used to estimate the hydrolysis rate of esterase. In vivo chlorophyll *a* fluorescence was measured in the FL3 detector (>650 nm). The fluorescence detector gathered information about extinction energy emitted by chlorophyll autofluorescence simultaneously for each cell. Cells were estimated as the area under the curve from a plot of cell number counted directly versus chlorophyll *a* fluorescence by FCM.

2.5. Microscopic observations of BCs and cyanobacteria

An optical microscope was used to observe the combination of BC particles and cyanobacteria. 0.5 g BC was added into 100 ml sterilised medium, which was inoculated with *Microcystis aeruginosa*. Images were taken 1 h after inoculation, and the interaction between BCs and cyanobacteria was recorded.

2.6. Statistical analyses

Each treatment of the experiments had three replicates. Data were presented as means ± standard deviation (SD). Statistical analyses were performed using SPSS 16 for Windows (Version 16.0). One-way analysis of variance (ANOVA) was performed followed by an independent-samples *t*-test to compare differences among control and treatment cultures. Significance was assumed at the five percent level.

3. Results and discussion

3.1. Characterisation of BCs

Selected physico-chemical properties of BCs used in this study are listed in Tables 1 and 2. FP and RP had lower C content and lower surface area than that of FC and RC. Energy dispersive X-ray spectroscopy (EDS) was used to determine that RP contained large amounts of K and Si, while FP contained large amounts of Si, Ca, Mg, Na, K, and Al (Ahmaruzzaman, 2010; Gupta and Sinha, 2008). The acid demineralisation process greatly increased C content, surface area and acid functional groups (Table 2). In particular, RP increased its surface area from 2.89 to 72.10 m^2 g^{-1}, its pore volume from 0.020 to 0.133 ml g^{-1}, its mean pore size from 13.88 to 64.31 nm, and its acid functional groups from 2.686 mmol g^{-1} to RC 3.741 mmol g^{-1}.

Based on the value of H/C, the maturity order of FC is higher than that of RC. RP (3.917 mmol g^{-1}) and RC (3.741 mmol g^{-1})

Table 1
Elemental compositions (CHN) and surface characteristics of BCs.

BC	Elemental composition (percent)			Atomic ratio		Surface area (m^2 g^{-1})	Mesopore	
	C	H	N	H/C	N/C		Porosity (ml g^{-1})	Average pore size (nm)
RP	2.02 ± 0.03	0.42 ± 0.03	0.15 ± 0.01	0.208 ± 0.011	0.074 ± 0.006	2.89 ± 0.55	0.020 ± 0.003	13.88 ± 1.24
RC	18.49 ± 0.69	0.71 ± 0.08	0.69 ± 0.06	0.038 ± 0.003	0.037 ± 0.002	72.1 ± 2.97	0.133 ± 0.016	64.31 ± 3.09
FP	10.47 ± 1.01	0.23 ± 0.04	0.18 ± 0.04	0.022 ± 0.001	0.017 ± 0.001	7.85 ± 0.48	0.032 ± 0.003	17.98 ± 1.41
FC	29.68 ± 2.38	0.26 ± 0.05	0.00 ± 0.00	0.009 ± 0.003	0.000 ± 0.000	21.01 ± 1.41	0.387 ± 0.024	20.53 ± 0.75