

Interactions between polycyclic aromatic hydrocarbons (PAHs)-degrading strain *Sphingomonas* sp. GY2B and nano bamboo charcoal

Xueqin Tao^{a,*}, Jin Wang^b, Xingchun Duan^{c,*}, Mengyao Zou^a, Jianjun Du^a, Jinlian Zhang^b, Guining Lu^b

^aCollege of Resources and Environment, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China, emails: xqtao@foxmail.com (X. Tao), mengyaozou_zhku@126.com (M. Zou), jianjundu@126.com (J. Du)

^bThe Ministry of Education Key Lab of Pollution Control and Ecosystem Restoration in Industry Clusters, School of Environment and Energy, South China University of Technology, Guangzhou 510006, China, emails: 201721042854@mail.scut.edu.cn (J. Wang), jinlian.zhang@scut.edu.cn (J. Zhang), GNLu@foxmail.com (G. Lu)

^cGuangdong Institute of Analysis, China National Analytical Center, Guangzhou 510070, China, email: 34753250@qq.com (X. Duan)

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ABSTRACT

As the global consumption of engineered nanomaterials such as nano bamboo charcoal (NBC) steadily increases, so does the potential for their release into the environment through human activity. In this study, the aggregation and dispersion of NBC, and its effect on GY2B, a polycyclic aromatic hydrocarbons (PAHs)-degrading strain of *Sphingomonas* sp., were investigated. Results showed that NBC aggregation was affected by both its concentration and GY2B, while the zeta potential of NBC was mainly influenced by GY2B and less so by its concentration. With the addition of NBC, the biodegradation rate of phenanthrene was enhanced while GY2B growth was inhibited initially, however, cell growth inhibition was attenuated and eliminated with NBC aggregation and sedimentation which adsorbed toxic metabolites, such as 1-hydroxy-2-naphthoic acid, eventually resulting in more favorable conditions. This work indicates that NBC has the potential to be used in environmental remediation.

Keywords: Nano bamboo charcoal; *Sphingomonas* sp.; Sedimentation; Environmental behavior; Biodegradation; Phenanthrene

1. Introduction

The *Sphingomonas* genera represents a new kind of functional microbial resource, with potential applications in environmental remediation. Many of its members, isolated from different geological areas, have been shown to degrade a wide range of polycyclic aromatic hydrocarbons (PAHs) [1,2] and related compounds, including phenol [3,4], dioxin [5], estrogen [6], azo dyes [7], and phthalic acid ester [8]. The *Sphingomonas* sp. strain GY2B which was isolated from contaminated soils, has been shown to efficiently use phenanthrene and related aromatic compounds, such as

naphthalene, 2-naphthol, salicylic acid, catechol, phenol, benzene, and toluene, as its sole carbon and energy source in mineral salts medium (MSM) [9]. There have been many reports on the relationship between functional microbes in the environment and indigenous bacteria [10–12], however, the relationship between functional microbes and their environmental surroundings, especially the relationship between functional microbes and nano particles in the environment, has not received the same level of attention [13,14].

With the rapid development of nanotechnology, nano materials, such as gold nanoparticles [15,16], silver nano-materials [17], organo-nano-clay [18], and nano bamboo charcoal (NBC) [19,20], have been widely used in many

* Corresponding authors.

industries including textiles, plastics, and paint. Nano materials will inevitably enter the environment during their production and use and could accumulate to a point where the health of humans and ecological systems are impacted [21–23].

As a new environmental-friendly and functional material, research on, and the developmental utilization of, bamboo charcoal has received much recent attention [24]. NBC is being used in a number of fields including textile [25], plastics [26], biomedicine [27], and QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation [28], but its nano form may harbor a potential risk to the environment. Empirical research on the effects of NBC, its mechanism of action, and related matters, is still in its infancy. Characterizing the dispersion and deposition of nanoparticles in the environment is critical to understanding their mechanism of transport and effects [29]. Due to the small size of nanoparticles, the unsaturated bonds on their surface have great surface activity, and because of their associated Van der Waals forces and static electricity, nano particles dispersed into a liquid medium can be easily absorbed onto various surfaces, including their own, which leads to aggregation [30]. NBC behavior in the environment (for example, dispersion, aggregation, and sedimentation in water) will be influenced by many factors [31,32], including the microorganisms present in the medium. The causal link between nano particles and their biological effects has not yet been established.

In this study, we investigated the settling of dispersed NBC in water and their effect on the growth of GY2B, a *Sphingomonas* sp. strain. The aim was to understand the transport characteristics of NBC in the environment and explore its potential for use in environmental remediation.

2. Materials and methods

2.1. Materials

GY2B, a PAHs-degrading strain of *Sphingomonas* sp., was isolated from crude oil contaminated soils collected at a site near the Guangzhou Petrification Company, China [9]. NBC (purity > 97%) was purchased from Shanghai Hainuo Coal Industry Co., Ltd., (China), and a stock solution of 10 g/L in distilled water was prepared and treated with ultrasound for 10 min before each use. Phenanthrene (purity > 98%) was purchased from Fisher Company (USA), and a stock solution of 10 g/L in cyclohexane was prepared and stored in a brown bottle at 4°C. All other chemicals used were of the highest purity available. The nutrient agar and MSM solution were prepared following a protocol adapted from Tao et al. [9].

Cell growth was estimated from the number of colony-forming units (CFU) appearing on agar plates incubated at 30°C. All media and solutions were prepared with distilled water and autoclaved at 121°C for 20 min. GY2B was activated for 2 d in MSM containing 100 mg/L phenanthrene prior to precipitation and degradation measurements.

2.2. SEM and particle size measurements

NBC samples in an ethanol solution were deposited onto a silicon wafer and dried at 80°C for 4 h. Scanning electron

microscopy (SEM) images of the samples were collected using a Carl Zeiss EVO LS10 (Germany) using an acceleration voltage of 10 kV [33]. After ultrasonic mixing for 15 min, a 3 mL sample of the NBC ethanol solution was placed in a quartz cuvette and particle size distribution was measured using a Malvern (UK) laser particle size analyzer (LB-550).

2.3. NBC sedimentation in the presence of GY2B

Determination of sedimentation rates of NBC in distilled water and MSM were carried out in 20 mL glass tubes. Aqueous solutions containing 2, 5, 10, 25, and 50 mg/L of NBC were prepared from the stock solution by dilution with distilled water and MSM, respectively. Two milliliters of GY2B was added to tubes containing 18 mL of the NBC solution. Each tube was covered with a rubber stopper and treated with ultrasound for 10 min, then placed in the dark at room temperature. Tubes with no bacteria added were used as a control. Tubes were taken out for measurement at each time point and 3 mL samples were taken to measure the absorbance at 480 nm. The extent of NBC sedimentation (S , %) was determined from the following:

$$S = \frac{A_0 - A_t}{A_0} \times 100\% \quad (1)$$

where A_0 is the initial absorbance and A_t is the absorbance after the sample has settled for t days.

2.4. Zeta potential measurements

The zeta potentials for NBC in both distilled water and MSM were measured with a Malvern Zetasizer Nano ZS. One milliliter of aqueous solutions containing 2, 5, 10, 25, and 50 mg/L of NBC were prepared as above (section 2.3 – NBC sedimentation in the presence of GY2B), sampled, and tested. Samples with no bacteria added were used as a control and all assays were carried out in duplicate.

2.5. GY2B growth in the presence of NBC

GY2B was exposed to NBC in MSM solutions. Twenty-seven milliliters of MSM, containing 100 mg/L of phenanthrene, was added to a series of 100 mL glass flasks. Three milliliters of activated GY2B culture and either 0.15 or 0.60 mL of NBC stock solution were added to the flasks which were then incubated in a shaker (150 rpm) at 30°C. Flasks containing no phenanthrene, NBC, or bacteria were included as controls and all assays were performed in triplicate.

2.6. Analytical methods

NBC's effect on GY2B growth in MSM was determined by exposing the microorganisms to different NBC concentrations and measuring the number of CFU that formed on nutrient agar [34].

Fifteen percent of NaCl (by weight) was added to the flasks to avoid emulsification and the phenanthrene was then extracted using an equal volume of cyclohexane. After allowing the sample to separate for 1 h, 5 mL of the

organic phase was taken and a certain amount of anhydrous sodium sulfate added. The phenanthrene in the cyclohexane solution was measured using the absorbance at 254 nm (OD_{254}) [35]. This method is quite linear for concentrations between 0.04 and 4.0 mg/L, as shown by the correlation coefficient (>0.999) between phenanthrene concentration and the OD_{254} . Control tests with varied phenanthrene concentrations were run to assess the recovery efficiency under extraction conditions, which was shown to be $>85\%$ for phenanthrene concentrations as low as 0.01 mg/L.

To identify the intermediate products of phenanthrene degradation, GY2B was grown in MSM with 100 mg/L phenanthrene as the sole carbon and energy sources, along with 200 mg/L NBC. Samples for UV-vis analysis were incubated for 0–120 h and prepared by centrifuging the culture medium at 11,000 rpm (4°C) for 20 min. The resulting supernatant was scanned from 200 to 500 nm using a Hitachi model U-3010 spectrometer (Japan).

2.7. Statistical analyses

SPSS 17.0 was used to perform the statistical evaluation of the results. The mean and standard values of triplicates were calculated and before being subjected to parametric statistical analyzes using a one-way analysis of variance (ANOVA), the data were tested for the assumption of normality of distribution and homogeneity of variance. All tested data satisfied the assumption.

3. Results and discussion

3.1. NBC characteristics

SEM images showed that NBC is of irregular shape, ranging in size from tens of nanometers to several microns (Fig. 1), and using the Malvern laser particle size analyzer, the average particle size was shown to be 401.9 nm.

3.2. NBC sedimentation in the presence of GY2B

Sixty-eight days of experiments were conducted to determine NBC sedimentation in both distilled water and

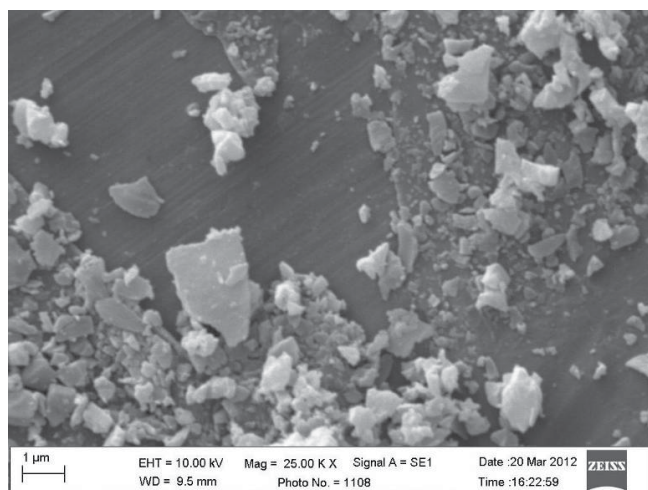


Fig. 1. SEM image of NBC.

MSM solutions. As shown in Fig. 2, the sedimentation of NBC in distilled water was about 90%, and in MSM was more than 99.9%. In both cases, the concentration of bamboo charcoal (2–50 mg/L) had little effect on sedimentation. These data indicate that in MSM solutions, NBC sedimentation was faster than in distilled water. This may be due to the existence of many inorganic ions in MSM solutions which could cause the diffusion double layer of the bamboo charcoal particles to be thinner, thus reducing the resistance of electric double layer. The surface potential was also reduced, which in turn means that the stability of the system has decreased [36]. When the many positive ions rush into the adsorption layer in the MSM system, the diffusion layer would completely disappear (after reaching an isoelectric state), and the electrostatic repulsion between the particles would disappear. Under these conditions, small particles would tend to coalesce, resulting in nano particles becoming micron aggregates which would accelerate the rate of sedimentation [37].

NBC sedimentation in distilled water in the presence of GY2B was more complex (Fig. 2). The results show that adding GY2B to the 2 mg/L NBC solution had little effect on final sedimentation, when adding to 5 and 10 mg/L NBC solutions sedimentation sped up, while its addition to 25 and 50 mg/L NBC solutions slowed the rate down. These results suggest that in distilled water, NBC concentration is a key factor affecting sedimentation rate in the presence of GY2B.

In contrast, NBC sedimentation in MSM was much simpler to assess (Fig. 2). NBC sedimentation in MSM in the presence of GY2B slowed down overall, and the decrease was correlated with NBC concentration, the higher the concentration, the greater the decrease in sedimentation rate. An underlying reason for this observation may be that the bacterial surface was negatively charged and could adsorb cations, resulting in a thicker double layer than was the case without bacteria [38]. The overall effect would be that NBC stability in MSM is increased in the presence of GY2B, resulting in slower sedimentation. The sedimentation photos (Fig. 3) of NBC with GY2B also showed that after a long period of settling in the MSM solution (68 d), the accumulation layer appeared to be thicker and looser than that seen in distilled water.

3.3. Effect of GY2B on NBC's zeta potential

The zeta potentials for NBC in both distilled water and MSM were measured (Fig. 4).

The zeta potential, which can be considered as the surface potential of the particles, directly affects the electrostatic potential energy between particles. The data suggested that the zeta potential of NBC in these two systems was influenced more by the presence of bacteria and less by the concentration of NBC. As shown in Fig. 4, pure GY2B had a low negative zeta potential (-14.15 mV in distilled water and -16.22 mV in MSM solution) and pure NBC particles had a high negative zeta potential (-36.02 to -39.19 mV in distilled water and -22.27 to -31.92 mV in MSM solutions). In water, NBC could remain in a stable suspension, mainly due to the higher electrostatic repulsion between the particles [39]. When the dispersed particles were smaller and the zeta potentials (positive or negative) were higher,

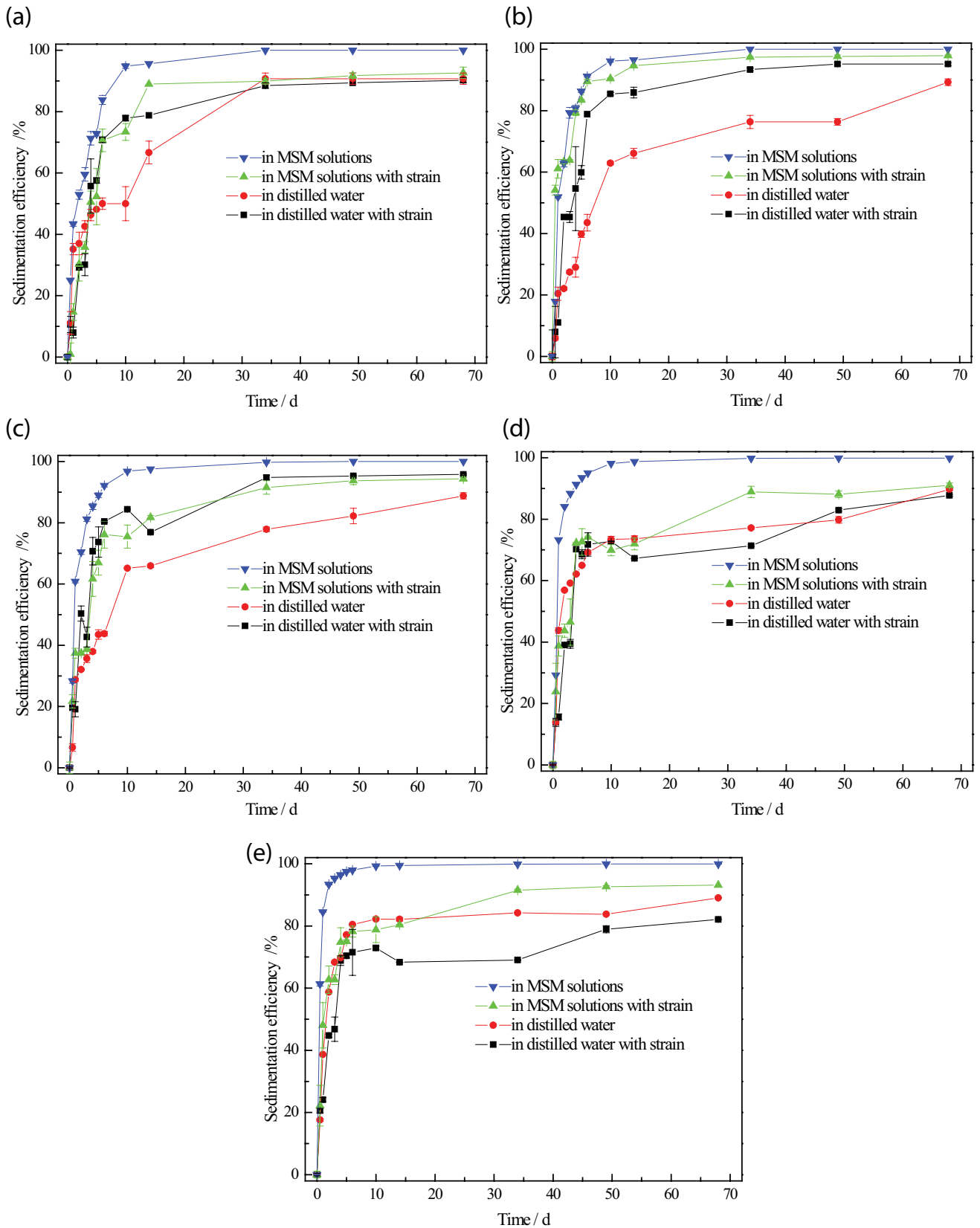


Fig. 2. Sedimentation of NBC concentrations in various systems. (a) 2mg/L NBC, (b) 5mg/L NBC, (c) 10mg/L NBC, (d) 25mg/L NBC, and (e) 50mg/L NBC.

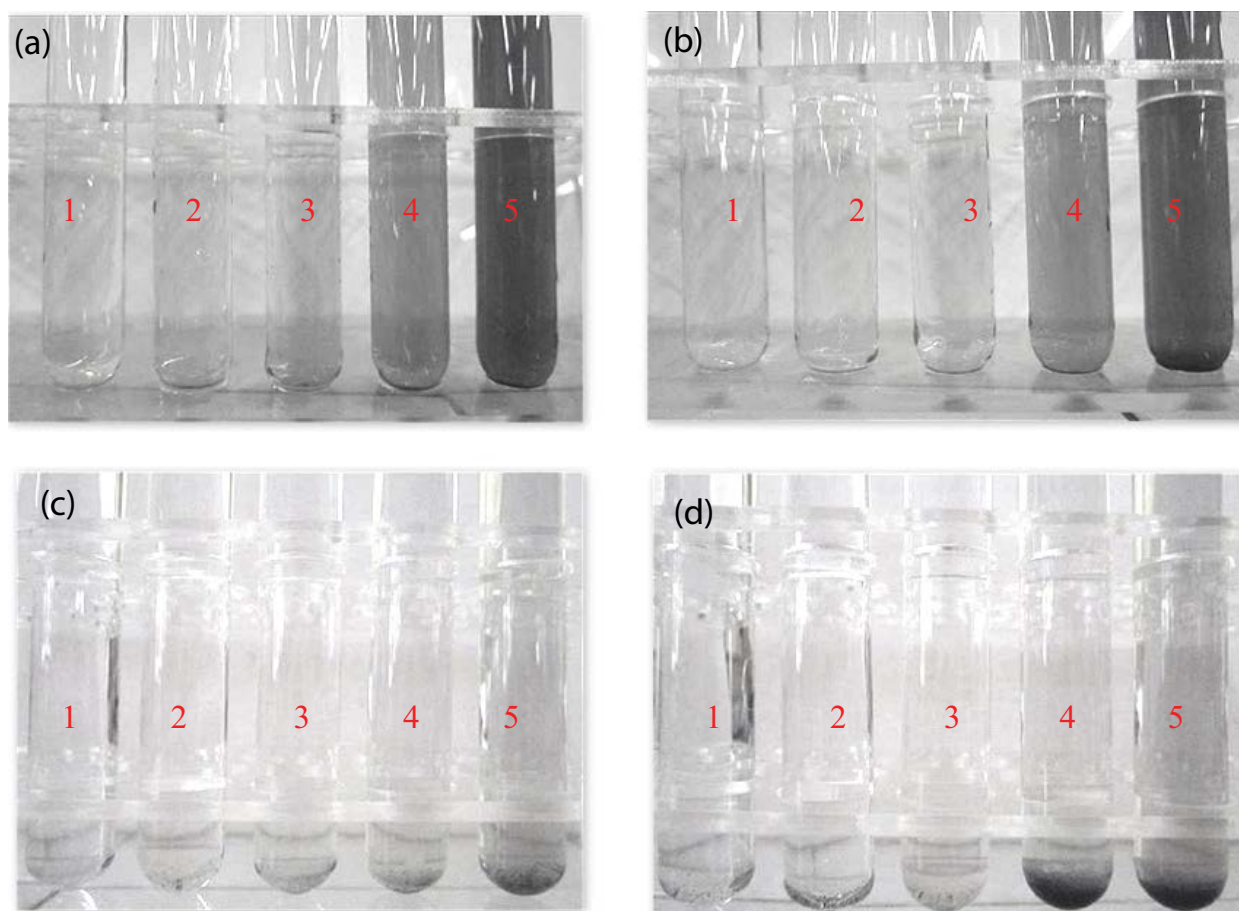


Fig. 3. Sedimentation photos of NBC with GY2B in distilled water and MSM. NBC concentrations were 2, 5, 10, 25, and 50 mg/L from tube 1 to 5. (a) 0 d in distilled water, (b) 0 d in MSM, (c) 68 d in distilled water, and (d) 68 d in MSM.

the system was more stable. Otherwise, if the zeta potential (positive or negative) decreased and the attractive force was larger than the repulsion force, then the favored result would be aggregation. In blank controls without GY2B, the zeta potentials of different concentrations of NBC in distilled water were much higher than those in MSM. This result was in agreement with the observation that NBC settled faster in MSM than distilled water.

The same measurement performed after the addition of GY2B to NBC solutions resulted in zeta potential of -14.15 to -15.97 mV in distilled water and -12.92 to -16.22 mV in MSM solutions. These zeta potentials were similar to those of GY2B alone and supported the conclusion that the zeta potentials of the system were mainly influenced by the presence of bacteria.

3.4. Effect of NBC on GY2B growth

NBC toxicity to GY2B was investigated with 50 and 200 mg/L NBC in MSM containing 100 mg/L phenanthrene. Fig. 5 shows the effect on GY2B growth during 120 h of exposure.

The greatest inhibition of growth occurred at 6 h followed by 12–72 h. After 72 h, NBC concentration appeared to be beneficial for GY2B growth. This observed difference

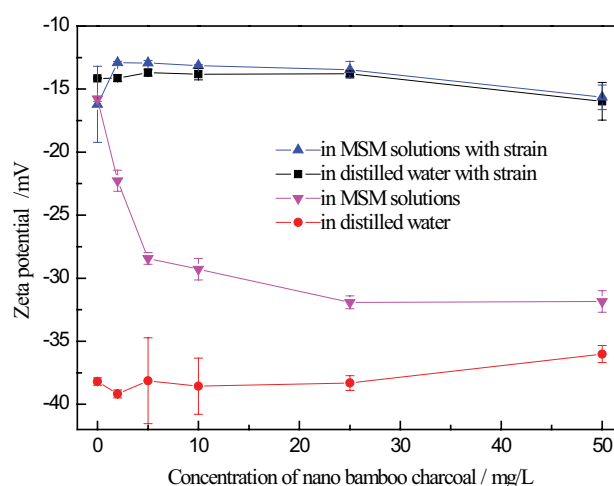


Fig. 4. Zeta potential of NBC in distilled water and MSM systems.

in growth may be caused by the characteristics of NBC and the bacteria. Other studies have shown that nano particles can directly damage bacterial cells [40]. At the beginning of the experiment (i.e., before 6 h), GY2B might have had

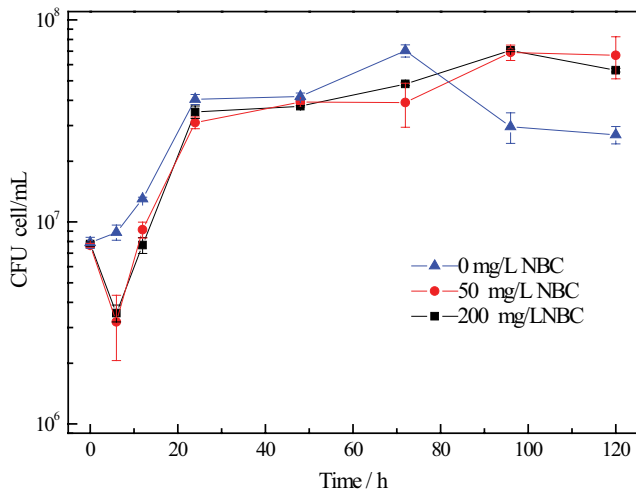


Fig. 5. Growth curve of GY2B incubated with NBC.

difficulty adapting to the culture conditions due to the direct damage caused by the NBC meaning that cell density was lower at the early time points than in the control. However, as time passes, the NBC gradually aggregates and the particle size increases, weakening its poisoning effect. After 72 h, once nutrients were consumed and toxic metabolites had accumulated in the medium, the culture mediums were no longer suitable for microbial growth, and so the cell density of the control gradually decreased. However, NBC may have adsorbed some toxic metabolites produced by the cells, which meant that the cell density of the experimental groups was maintained in a basically stable state and thus they continued their slow growth, exceeding that of the control group after 72 h. The adsorption function of NBC is discussed below.

3.5. Effect of NBC on phenanthrene degradation by GY2B

Phenanthrene degradation by GY2B was examined in a 200 mg/L solution of NBC. As shown in Fig. 6, the initial degradation of phenanthrene was greatly increased during the first 18 h with the addition of NBC. About 94% phenanthrene was degraded at 18 h and it was almost completely degraded at 48 h.

The data in Fig. 5 shows that NBC had a toxic effect on the growth of GY2B, leading to a decrease in cell density in the first 6 h of exposure. However, NBC had no adverse effect on phenanthrene degradation by GY2B. On the contrary, NBC promoted degradation when compared with the control. Fig. 6 shows that about 4.5%–8.0% of the phenanthrene was strictly adsorbed by NBC and difficult to extract with cyclohexane. In general, phenanthrene solubility in water is only 1 mg/L, but this would increase in a solution containing nanoparticles [41]. Only the dissolved phenanthrene could be utilized by microorganisms as a carbon and energy source, which may be one reason why NBC could promote phenanthrene degradation.

In order to further clarify the influence of NBC on phenanthrene biodegradation, the culture supernatants were analyzed using UV-visible spectroscopy. The absorption curves of the control sample (Fig. 7a) reveals

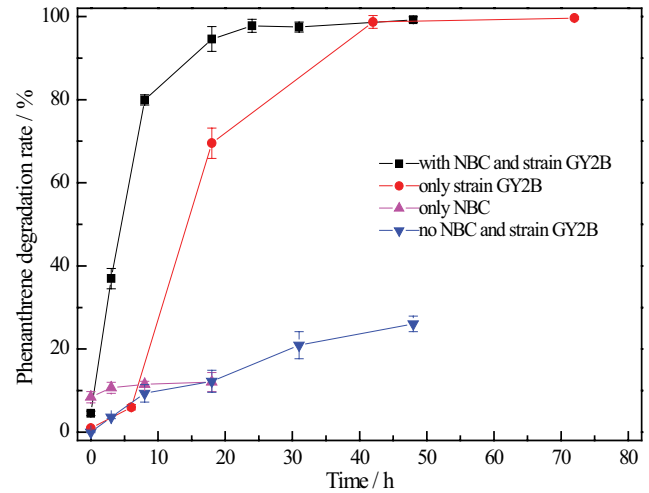


Fig. 6. Phenanthrene removal under different conditions.

peaks at about 250 nm, a characteristic absorption peak of phenanthrene. At 6 h the characteristic absorption peaks of 1-hydroxy-2-naphthalene acid, a previously identified phenanthrene degradation intermediate, appeared [9]. The absorption peaks reached a maximum at 12 h and then gradually decreased, reaching a level at 72 h that was almost coincident with the level at 96 h. In Fig. 7b, the peaks of the spectra of the sample after adding 50 mg/L NBC were very similar with those seen in the control at each wavelength, however, the absorbance values were lower than those of the control. After adding 200 mg/L NBC, weak 1-hydroxy-2-naphthalene acid peaks were only found in the 12 h sample (Fig. 7c). The absorption curves at later times did not differ significantly from those at 0 h and were even lower in some cases. These results indicated that the NBC had adsorbed phenanthrene and/or the metabolic intermediates. This was consistent with our previous observations that the cell density was higher following the addition NBC than in the control group after 72 h.

4. Conclusions

Nanoparticles in natural waters exhibit complex properties in the environment. The following conclusions have arisen from this study: (1) sedimentation rate of NBC in MSM was faster than that in distilled water because of the large amount of inorganic ions in the MSM. Also, rapid sedimentation rates reduce both the resistance of the electric double layer and the surface potential, (2) NBC concentration is the key factor affecting its sedimentation when GY2B is present, while the zeta potential is mainly affected by bacteria, (3) phenanthrene biodegradation was enhanced by GY2B in the presence of NBC, even though NBC could initially inhibit GY2B growth. The inhibition of cell growth could be attenuated and eliminated with the aggregation and sedimentation of NBC which adsorbed toxic metabolites such as 1-hydroxy-2-naphthoic acid, resulting in more favorable conditions for GY2B growth. Future research should focus on assessing the fate of NBC in the aquatic environment and its potential application in environmental remediation.

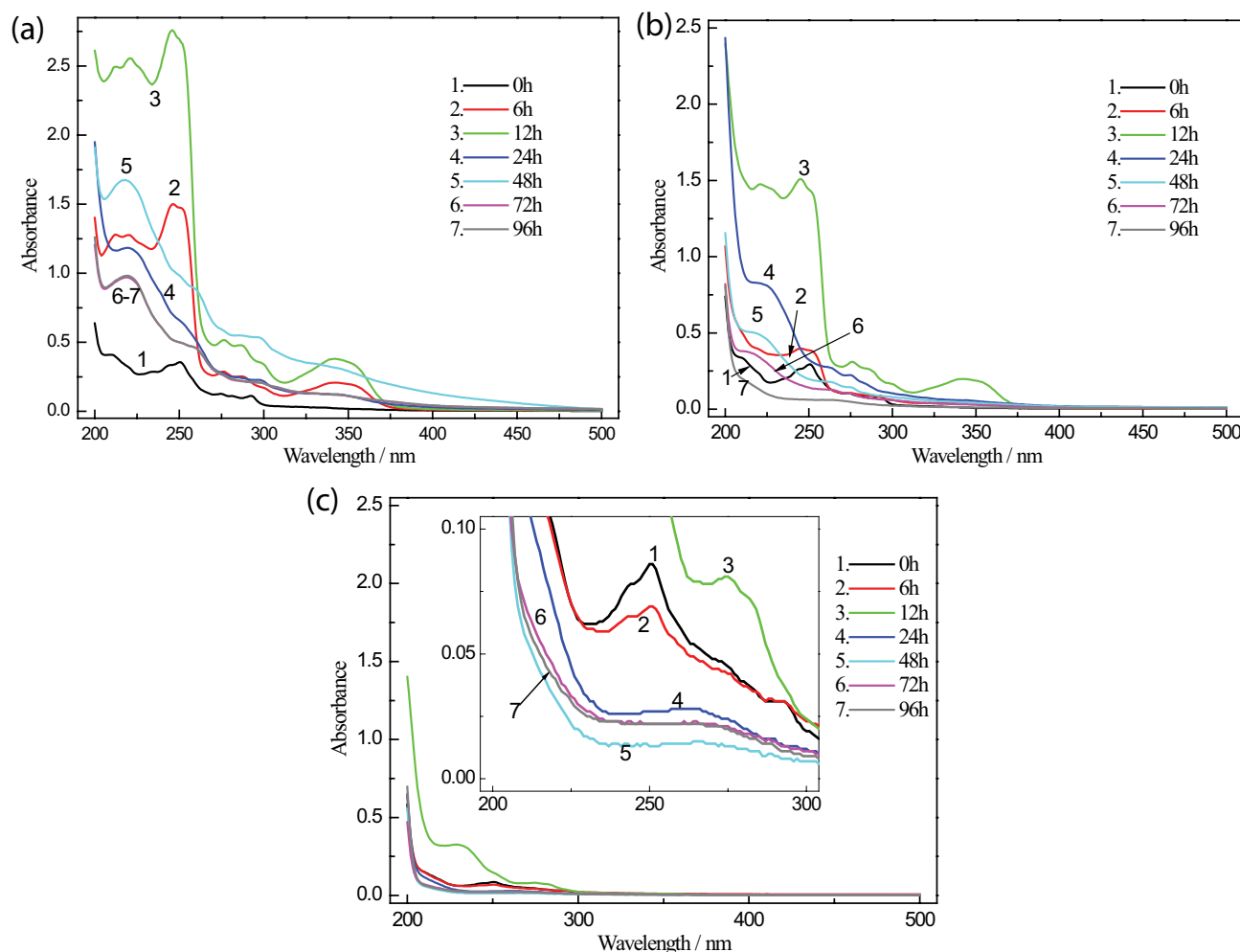


Fig. 7. UV-vis spectra of culture supernatants from 0 to 96 h with NBC (a) control (no NBC), (b) 50 mg/L, and (c) 200 mg/L NBC.

Acknowledgments

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